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Regulation of Toxoplasma Motility by Calcium-Dependent Protein Kinases

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by

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ABSTRACT OF THE DISSERTATION

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Apicomplexans are protozoan parasites of animals, which must penetrate host cells to find a niche in which to replicate. To humans, they represent ubiquitous and deadly eucaryotic pathogens, including *Toxoplasma gondii* and *Plasmodium spp.*, the etiological agents of toxoplasmosis and malaria, respectively. A common mechanism enables these parasites to penetrate biological barriers and invade host cells actively, through a process termed gliding motility. This type of motility, unique to apicomplexans, relies on the directional translocation of adhesins via an actomyosin motor complex anchored in a vesicular network underlying the plasma membrane of the parasite. A variety of stimuli can trigger calcium increases in the parasite cytoplasm initiating motion, in part by regulating the secretion of adhesins from specialized organelles, called micronemes. Our studies investigate how these calcium signals are transduced to regulate *T. gondii* motility. Apicomplexans, like other chromalveolates and plants, possess calcium-dependent protein kinases (CDPKs) that are directly activated by calcium binding and have been proposed to participate in the transduction of calcium signals. A number of CDPKs are conserved among apicomplexans, and showed distinct subcellular localizations upon tagged expression in *T. gondii*, consistent with roles in different calcium-activated cellular pathways. Using a combination of chemical and genetic approaches we demonstrated that two of the conserved

CDPKs are required for motility at different stages in the *T. gondii* life cycle. Generating a conditional knockout, we showed that TgCDPK1 is required for microneme secretion during egress and invasion. The essential role of this kinase was further supported through the use of bulky pyrazolo [3,4-d] pyrimidine (PP) analogues that inhibit TgCDPK1, and mirrored the effects of the conditional knockout. The specificity of these compounds is conferred by the expansion of the ATP-binding pocket in TgCDPK1 caused by the presence of a glycine at a key position called the 'gatekeeper', a feature unique among parasite kinases. Mutating this residue to a methionine made TgCDPK1 resistant to the inhibitors, and enabled us to mutate the gatekeeper residue of a related kinase, TgCDPK3, rendering it sensitive to inhibition by PP analogues. This chemical-genetic strategy allowed us to implicate TgCDPK3 in the initiation of motility during egress, but demonstrate that its function is dispensable during invasion. Together, these observations provide the first evidence that related CDPKs regulate distinct signaling pathways, which distinguish the signaling events governing motility during egress and invasion by *T. gondii*. We also attempted to further understand the role of TgCDPK1 by examining its cellular targets. This process was also facilitated by the atypical ATP-binding pocket of TgCDPK1, which was able to bind bulky ATP analogues that allowed us to track its direct targets. This approach has allowed us to identify a number of putative TgCDPK1 targets. One such target, a dynamin-related protein, is phosphorylated *in vivo* in a CDPK1-dependent manner, consistent with a role in the regulation of motility. Together these observations provide a foundation for further characterization of CDPK signaling and the regulation of parasite motility. In particular, the chemical-genetic approaches adapted to parasites in these studies, represent a systematic means to dissecting these essential pathways in apicomplexans.

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CHAPTER I

Introduction

PREFACE

This chapter was composed entirely by Sebastian Lourido. Comments from David Sibley are incorporated into the final version presented here.

A PARASITE OF DOUBTFUL NATURE

Diverse relationships link individual species into the collectives we recognize as ecosystems. The relationships we are most familiar with are parasitic due to the economic and social costs of parasites on agriculture and human populations. The competing interests of the parasite and its host often drive remarkable adaptation and consequent specialization, exemplified in the extreme by intracellular parasites. Many of these parasites require different hosts to complete their life cycle, a behavior termed heteroxenous, and restrict their sexual cycle to a single host, defined as the definitive host. Parasites belonging to the genus *Plasmodium*, the etiological agents of malaria in a wide range of vertebrates, carry out heteroxenous life cycles between mosquito definitive hosts and vertebrate intermediate hosts. Individual *Plasmodium* species are restricted to one or a few mosquito and vertebrate species, demonstrating yet further specificity in the tissues that can be infected by each developmental stage in the parasite life cycle. Similar specificity has also been described for the genus *Eimeria*, for which dozens of host-specific species had been catalogued by the end of the 19th century (Wenyon, 1926a). This paradigm in parasitism would be challenged by the discovery of the genus *Toxoplasma*.

The earliest descriptions of *Toxoplasma* occurred nearly concurrently by two investigators: in tissues from north African rodents called gundis by Charles Nicolle (1907), and in Brazilian rabbits by Alfonso Splendore (1908). Although both researchers initially suggested the organisms belonged to the genus *Leishmania*, further investigation led to the conclusion that a new genus should be created and called *Toxoplasma*, for the Greek *toxon* describing the parasite's crescent shape. Soon thereafter, others started describing similar parasites in a variety of animals including mice, rats, guinea-pigs, sparrows, vultures, dogs, and others (Wenyon, 1926a). In the tradition of other protozoan genera, the *Toxoplasma* isolates were assigned species-specific names according to the host bearing them. However, studies soon demonstrated the remarkable ability of different *Toxoplasma* isolates to infect

a variety of hosts. For example, the organism isolated from gundis was shown to infect rabbits, guinea-pigs, dogs, mice, moles, pigeons and sparrows. Compounded with the lack of morphological distinctions, the identity of the different species was brought into question. Intriguingly, at the institute where Nicolle was working, the parasite could only be identified in animals that had been kept in captivity. "Of 400 animals examined within a month of capture, only two were infected, while of seventy-one examined after a month of confinement, thirty-three were infected." (Wenyon, 1926b). This was interpreted to mean that even the index case for the genus might have originated from an altogether different organism. It was not until the 1940's with the development of an immunological assay (Sabin & Feldman, 1948) that epidemiological data could be gathered supporting the view that all the *Toxoplasma* isolates belonged to a single species, *Toxoplasma gondii*. We now know from sequence analysis of field isolates that *T. gondii* strains are highly similar, and although certain clonal lineages are more prevalent in different geographic regions and hosts, no restrictions in their host tropism or ability to mate supports the notion of different species (Su *et al*, 2012).

It should be recognized that the remarkably broad host range of *Toxoplasma* remains an oddity among the parasitic protozoa, and perhaps most infectious agents. Its ubiquity among wild and domestic animals, while facilitating its initial discovery precluded the identification of its sexual cycle in felids, until 1970 (Frenkel *et al*, 1970), and the full recognition of its relation to *Eimeria* and other coccidians. Its ease of culture has enabled a greater understanding of the cell biology of apicomplexans as a whole. In particular *T. gondii* has served as a model for the type of motility unique to apicomplexans, which lies at the heart of host cell invasion by these parasites, and perhaps forms one of the pillars of their evolutionary success.

APICOMPLEXAN BIOLOGY: A COMMITMENT TO PARASITISM

The phylum Apicomplexa is comprised from thousands of obligate animal parasites. Their diversity represents millennia of coevolution with their animal hosts, as well as increasingly complex modes of parasitism involving closer associations with the host cells they derive sustenance from.

The most related free-living organisms to apicomplexans, colpodellids and dinoflagellates, parasitize larger protozoa by a process termed myzocytosis, whereby specialized organelles are used to penetrate the plasma membrane of their prey, and extract portions of cytoplasm to digest. Gregarines, apicomplexans that infect a varied array of invertebrate hosts, display similar feeding behaviors, and it is intriguing to speculate that this rudimentary association might represent the ancestral ability that enabled apicomplexans to become obligate parasites (Barta & Thompson, 2006). This notion is supported by the presence of microtubular structures in the feeding organelles of colpodellids and dinoflagellates, reminiscent of the conoids found in apicomplexans (Cavalier-Smith & Chao, 2004). Another feature shared by colpodellids and apicomplexans is the flattening and fusion of the alveoli, to create a continuous inner membrane complex underlying nearly the entirety of the plasma membrane. It has been speculated that flattening of the alveoli is necessary to create the continuous area required for efficient gliding motility, which is hypothesized to enable penetration of the pray's membrane by colpodellids, and which apicomplexans use to penetrate and invade host cells (Cavalier-Smith & Chao, 2004). Although colpodellids move through the use of flagella, gliding motility has become the exclusive means of motility for apicomplexans, with the exception of their male gametocytes, which develop flagella (Sinden *et al*, 1976; Ferguson *et al*, 1974).

Apicomplexan cell cycles vary greatly to incorporate adaptations to different hosts. Generally, all apicomplexans proliferate asexually before generating gametocytes, which pair to create a zygote. The zygotes of some species produce environmentally resistant

oocysts, which may be excreted from one host and ingested by another, as a means of dispersion. Sporozoites develop within the oocyst and emerge to give rise again to the asexual cycle. Gliding motility enables sporozoites to gain access to a variety of niches within the host, where the parasites can differentiate into feeding forms, called trophozoites, which often bear little resemblance to sporozoites or to the trophozoites of different species. Gregarines illustrate the most extreme examples of this transformation, perhaps as adaptations to different modes of nutrient acquisition. Many of the gregarines that live in the intestines of insects have trophozoites with highly convoluted membranes, which probably serve to increase the surface area for nutrient acquisition (Leander, 2008). Other gregarines cross the intestinal epithelium as sporozoites before transforming into large trophozoites within the coelom. Their trophozoites often have a bifurcated or flattened appearance and display peristaltic movements that may aid fluid flow over their highly corrugated surfaces (Leander, 2008). These specialized adaptations are an alternative to myzocytosis, which can also be found in many gregarines and is thought to be the ancestral form of nutrient acquisition from its presence in colpodellids and many of the earliest gregarine radiations (Leander, 2008; Barta & Thompson, 2006). Most of the myzocytic gregarines only partially invade their host cell, creating a sophisticated organelle that enables more permanent attachment and nutrient acquisition from the host (Barta & Thompson, 2006). It has been speculated that this structure is related to the feeding organelle of members of the genus *Cryptosporidium*. If this is true, it may represent an intermediate stage in the development of completely intracellular trophozoites since *Cryptosporidium* trophozoites remain epicellular but surrounded by the cell membrane of the host cell (Barta & Thompson, 2006). However, it is also possible that many of these features represent convergent evolution, which will require more phylogenetic information to resolve. Intracellular parasitism may be advantageous to the avoidance of immune responses, as well as to the spread beyond gut tissues in vertebrate hosts. The ability to invade tissues

beyond the gut tract permits the development of new routes of spread including vertical transmission and many heteroxenous life cycles. For many coccidian parasites vertical transmission can be a primary route of spread, as is the case for *Neospora caninum* within some cattle herds (Davison *et al*, 1999). Other apicomplexans rely on heteroxenous cycles that require systemic spread within the vertebrate host. This may involve infections of erythrocytes by haemosporidia like *Plasmodium spp.* or leukocytes by piroplasms, which are required for the infection of hematophagous definitive hosts. Similarly, apicomplexans of carnivores like *T. gondii* have evolved to infect the pray of their definitive hosts as a means of spreading. More recently *T. gondii* has developed the additional ability, unique among the coccidians, to bypass its sexual cycle by developing oral infectivity among intermediate hosts (David Sibley, 2003).

These examples, along with the apicomplexan phylogeny suggest that concomitant with the ability to fully invade host cells, many apicomplexans gained access to novel niches, and developed an ability to engage intermediate hosts in heteroxenous cycles (Barta, 1989). Many adaptations have enabled the apicomplexans of vertebrates to become intracellular. These adaptations are reflected by changes in physiology, cellular morphology and cellular signaling observed in parasitized cells. How apicomplexans modify their host cells remains an active area of research. Although specialization their intracellular niche, restricted certain aspects of the apicomplexan cycle, it also appears to have opened the possibility of new routes of infection, which has made these parasites so successful in spreading among vertebrates.

THE LIFE CYCLE OF *TOXOPLASMA GONDII*: CHOOSE YOUR OWN ADVENTURE

T. gondii belongs to a group of apicomplexans known as coccidians, characterized by undergoing sexual replication in the intestinal epithelium of their definitive vertebrate hosts to produce environmentally resistant stages called oocysts. Most coccidia spread by a strict fecal-oral rout, whereby the oocysts are ingested by another definitive host that can once again produce oocysts. However, the oocysts of *T. gondii* and other related coccidians can infect intermediate herbivore hosts and give rise, asexually, to tissue cysts that can infect their carnivorous definitive hosts. In this way *T. gondii* belongs to the group of tissue-cyst forming coccidia, which are distinguished from the true coccidia that are restricted to the gut of their host. Unique among the coccidians, the tissue cysts of some *T. gondii* strains can also orally infect other intermediate hosts, with important consequences to the spread of these parasites among a wide variety of animals (David Sibley, 2003). Therefore, unlike most coccidians, different routes of infection might serve to perpetuate *T. gondii* infections within different animal populations and environments.

Sexual cycle in felines

For many decades after the discovery of *Toxoplasma* in warm-blooded animals throughout the globe its life cycle remained elusive, leaving the rout of infection and its relation to other parasitic protozoa unresolved. With the discovery of the feline definitive host in the 1970's, many of the features common to all coccidia were also described for *T. gondii*, including the characteristic oocysts and the development of male and female gametes in the intestinal epithelium (Frenkel *et al*, 1970; Ferguson *et al*, 1974).

Cats could be infected by feeding on infected mice, and produced oocysts three to five days following infection for a period of one to two weeks. A number of morphologically distinct stages have been described in the cat following tissue cyst ingestion (Dubey &

Frenkel, 1972; Speer & Dubey, 2005). These stages are regarded as a period of asexual replication and differentiation on the path to generating the male and female gametes. Once formed the female gametes are spherical and remain near the villus edge of the infected epithelium. The male gametocytes are small with almost no cytoplasm and an elongated nucleus crowned by two apical flagella (Dubey & Frenkel, 1972; Ferguson *et al*, 1974). The motile male gametocyte is thought to penetrate and fertilize the female, forming a zygote that eventually matures to an oocyst.

Cats can also be infected, although with reduced efficiency, by ingestion of the rapidly replicating stages in the intermediate host (tachyzoites), and by mature oocysts. The period of time required to observe oocysts in the feces of infected cats (prepatent period), was longer for tachyzoite infections compared to tissue cyst infections, and longer still for oocyst infections. Differences in the prepatent period were used to postulate a cyclic development of the parasite (Frenkel *et al*, 1970).

Oocysts

An infected cat can produce millions of oocysts in a matter of days (Dubey & Frenkel, 1972), and once sporulated the oocysts can survive under mild conditions for months to years (Dubey, 1998b). Consequently, contamination of food and water with *T. gondii* oocysts is a major source of infection for animals and humans. In a field study comparing swine farms in Illinois, vaccination of cats to prevent oocyst shedding was correlated with a drop in seroprevalence in the field mice and pigs over the course of a few years (Mateus-Pinilla *et al*, 1999). Therefore, transmission through oocysts appears to be a primary means of perpetuating the *T. gondii* life cycle within many populations, despite the alternative routes of infection discussed above.

Once excreted, coccidian oocysts undergo sporulation, which involves two or more rounds of cell division to generate infectious sporozoites. The number of sporozoites

generated, as well as the overall structure of the sporulated oocysts is diagnostic of the species and has been widely used for classification (Todd & Ernst, 1977). The oocysts of *T. gondii* were shown to contain two sporocysts, each harboring four sporozoites (Frenkel *et al*, 1970). Following ingestion, sporozoites are released and invade the intestinal epithelial cells of the new host.

Asexual cycle in the intermediate host

Although many coccidians are monoxenous and spread strictly through the fecal-oral route, *T. gondii* and related coccidians have adapted to heteroxenous life cycles that involve a carnivore definitive host and a herbivore intermediate host (David Sibley, 2003). Within the intermediate host, sporozoites can differentiate into asexually replicating stages called merozoites, and infect other tissues throughout the animal's body. The merozoites of *T. gondii* are categorized as either tachyzoites or bradyzoites, for the Greek referring to their rapid or slow replication, respectively. As tachyzoites invade host cells, they form a parasitophorous vacuole, within which they replicate. *T. gondii* replicates primarily through endodiogeny, where DNA replication is immediately followed by nuclear division and cytokinesis. In the cat it can sometimes undergo endopolygeny, where multiple rounds of DNA replication and nuclear division can precede cytokinesis (Figure 1; Mehlhorn & Frenkel, 1980; Vaishnava & Striepen, 2006). Following multiple rounds of replication tachyzoites synchronously egress from the host cell to infect new cells (Figure 1). Recursive rounds of tachyzoite replication are responsible for the tissue disruption characteristic of toxoplasmosis. Under certain conditions, and in response to immune pressure, tachyzoites differentiate into bradyzoites and this latter stage gives rise to tissue cysts in a variety of tissues throughout the body of the intermediate host (Figure 1; David Sibley, 2003).

Spread of the asexual stages may give rise in some cases to vertical transmission, which may be asymptomatic or cause birth defects and in some cases abortion (Wong &

Remington, 1994). Although not widely recognized as a major source of infection, vertical transmission can be an important route for related coccidians (Davison *et al*, 1999), and has been demonstrated to occur for up to ten generations under experimental conditions in *T. gondii*-infected rodents (Beverley, 1959), although the latter has not been replicated since the original report. The asexual stages can also differentiate into slow replicating forms that create tissue cysts that may persist for the life of the host. Both asexual stages described here for the intermediate host can also occur in the feline host, and in kittens the systemic spread of asexual stages can cause severe disease (Dubey & Frenkel, 1972).

Tissue cysts

As described above, under specific conditions the asexual stages in the intermediate host can differentiate into bradyzoites that form tissue cysts. Bradyzoites can be distinguished by changes in transcription (Radke *et al*, 2005) and subtle ultra-structural rearrangements (Mehlhorn & Frenkel, 1980). The vacuole in which the bradyzoites reside changes significantly, accumulating a thick matrix on its surface that contains chitin, glycoproteins, and other factors of host and parasite origin (Sullivan & Jeffers, 2012). The size and shape of the tissue cyst can vary widely depending on the tissue where it forms and the number of bradyzoites it harbors (Sullivan & Jeffers, 2012). Tissue cysts can form in any tissue, but are most prevalent in neural and muscular organs like the eye, the brain and the heart (Petersen & Dubey, 2001). Analysis if tissue cysts in brain tissue shows that they remain within the host cell (Mehlhorn & Frenkel, 1980), which likely contributes to their persistence for the life of the host, even in the presence of adaptive immunity (Sullivan & Jeffers, 2012).

Bradyzoites have been shown to be more resistant than tachyzoites to a variety of stresses including osmotic changes and pepsin treatment, although sensitivity to these treatments can vary depending on the source of the tissue cysts (Dubey, 1998a). Compared

to oocysts and tachyzoites, bradyzoites are extremely infectious to cats and ingestion of just a few can lead to infection (Dubey, 2001). Although tissue cysts are more infections to cats than mice (Dubey, 2001), their ability to infect other intermediate hosts distinguishes *T. gondii* from all other coccidia. Consumption of meat containing tissue cysts is thought to be an important source of human infection. Although infections from oocysts and tissue cysts are clinically indistinguishable, epidemiological studies suggest that consumption of under-cooked or raw meat is correlated with seropositivity (Petersen, 2007). Tissue cysts can also provide a source for reactivation, and chronically infected cats are known to produce oocysts after challenge infection (Dubey, 1976).

Human toxoplasmosis

Among humans, seroprevalence varies widely between regions, with an estimated 25% of people worldwide being chronically infected (Hall *et al*, 2001). During acute infection, parasites can disseminate throughout the host due to their ability to invade virtually any nucleated cell and traverse biologically restrictive barriers like the intestine, blood-brain, blood-retinal and placental barriers (Barragan & Hitziger, 2008). Primary infections during pregnancy can therefore be transmitted to the fetus causing mental retardation, hearing loss, blindness and, in severe cases, abortion (Wong & Remington, 1994; Montoya & Liesenfeld, 2004). However, 90% of acute infections will go unnoticed in otherwise healthy individuals, leading to a self-limiting infection that induces the formation of tissue cysts that persist for the life of the host.

Chronic infections with *T. gondii* can recrudesce in immunocompromised individuals, including those suffering from AIDS, and organ transplant recipients. Prior to the availability of anti-retroviral therapy, toxoplasmosis was an AIDS-defining illness and a major HIV-related complication in countries with a high prevalence of *T. gondii* (Pereira-Chioccola *et al*, 2009). Heart transplant patients are particularly at risk of

infection due to the disproportionate tissue cyst burden in the heart compared to other transplanted organs. Risk of infection can be as high as 75% when a seronegative patient receives a heart from a seropositive donor, which can be common in countries with high prevalence of chronic infection (Derouin et al., 2008; Gallino et al., 1996). Lack of immunity or immunosuppression can lead to production of tachyzoites from the tissue cyst and disseminated infection. Repeated rounds of host cell invasion and replication causes tissue damage, which underlies the pathogenesis of *T. gondii*, and leads to myocarditis, encephalitis or pneumonitis.

The association between *T. gondii* infection and behavioral changes in mice (Vyas *et al*, 2007), has lead to significant speculation about the role of *T. gondii* in human neurological disease. A number of studies have established *T. gondii* seropositivity as an intermediate risk factor for schizophrenia (Torrey *et al*, 2012). Seropositivity has also been correlated with an increase in suicide rates (Ling *et al*, 2011) and affinity for the smell of cat urine (Flegr *et al*, 2011). However, given that factors affecting behavior can predispose individuals to *T. gondii* infection, it is difficult to establish causality in any of the studies described. Furthermore, comparing the high infection rates with the low incidence of these diseases suggests that in humans chronic infections are predominantly asymptomatic.

ANATOMY OF THE ZOITE: MORPHOLOGY AND FUNCTION OF APICAL ORGANELLES

Given their remarkable specialization to different hosts and niches, it is not surprising that the trophozoite stages of many apicomplexans vary dramatically. However many other stages, particularly those undertaking host cell invasion or the penetration of restrictive biological barriers, share a number of common features. Even before its relationship to other apicomplexans was illuminated by the discovery of its sexual cycle, *T. gondii* became a model for much of the ultra-structural characterization of these motile stages, collectively referred to as zoites. The following discussion will therefore place emphasis on the *T. gondii* zoite, and in particular the tachyzoite, which can be propagated in cell culture and is responsible for the tissue destruction that causes pathology.

All zoites are highly polarized cells, reflecting the directionality of their movement. Their shapes range from spherical to vermiform, with sizes ranging 1-20 m, although the trophozoite stages of many gregarines will dwarf this measure (Leander, 2008). *T. gondii* tachyzoites are bow-shaped and measure approximately 2 m wide by 7 m long (Figure 2). Like most alveolates, the plasma membrane of apicomplexan zoites is subtended by a series of vesicles called the pellicle or inner membrane complex (IMC). The IMC vesicles are flattened and fused under most of the parasite surface, unlike the analogous vesicles in other alveolates, leaving only the apical and posterior poles open (Morrissette *et al*, 1997). Microtubules extend beneath the IMC for most of the length of the zoite emanating from a ring-shaped MTOC at the apical end, and are thought to confer the elongated cell shape (Morrissette & Sibley, 2002). A number of specialized structures and organelles, important for host cell invasion, characterize the apical end, including polar rings, conoid, rhoptries and micronemes (Figure 2). Some of these features vary between species, and during development. In particular, secretory organelles, which include the apical rhoptries and micronemes, and the generally distributed dense granules, can vary in number during

development (Dubremetz & Ferguson, 2007). It is the interplay of all these cellular structures that permit zoites to invade host cells, which is ultimately the function of the zoite: to carry the genetic information from one site of replication to another.

Most apicomplexan parasites of mammals replicate inside host cells, following invasion, within a specialized compartment called the parasitophorous vacuole. The process of invasion has been best studied in *Plasmodium spp.* and *T. gondii*, where the zoites reorient to contact the host membrane with their apical ends, and proceed to make a tight junction, through which the parasite moves into the nascent parasitophorous vacuole (Figure 3). This tight junction (also known as the moving junction) was first observed in *P. knowlesii* merozoites invading erythrocytes, and consisted of the tight apposition of the parasite and host plasma membranes, with a ring of electron dense material in the adjacent host cytoplasm (Aikawa *et al*, 1978). This junction has since been shown to originate from the interaction of secreted proteins of rhoptry and micronemal origin, in both *Plasmodium spp.* and *T. gondii* (Besteiro *et al*, 2009; Richard *et al*, 2010). This moving junction is thought to exclude host proteins from the forming parasitophorous vacuole (Mordue *et al*, 1999), although this compartment is primarily derived from the host plasma membrane (Suss-Toby *et al*, 1996). Instead, the nascent vacuole is coated and remodeled by secreted parasite proteins to generate a niche within which these parasites can replicate. The paucity of research into invasion by other apicomplexans precludes the generalization of these mechanisms, though analysis of invasion by *Cryptosporidium parvum* suggests that gliding motility plays a similar role in this distantly related apicomplexan (Wetzel *et al*, 2005). In contrast, *Theileria* sporozoites, as will be pointed out below, do not use gliding motility to invade host cells and instead rupture the parasitophorous vacuole after invasion and replicate in the host cytoplasm (Shaw, 2003). This aberrant form of invasion seems to be a unique adaptation of *Theileria spp.*, but suggests that the formation of a parasitophorous vacuole suitable for replication requires gliding motility. Curiously, *Babesia spp.*, a related

piroplasm, does appear to use gliding motility to invade host cells, but also lyses the parasitophorous vacuole and replicates in the cytoplasm (Hines *et al*, 1995), suggesting that this might have been a prior adaptation to dispensing with gliding motility for entry.

Organelles of Endosymbiotic Origin

Most apicomplexans possess three genomes: the nuclear genome, the mitochondrial genome, and the apicoplast genome. The latter two represent organelles that originated from endosymbiotic events, where an invading or engulfed organism was incorporated into the host cell and passed along into its progeny. These organelles are not directly associated with invasion, but form such an integral part of the apicomplexan cell that they deserve mention.

Mitochondria, when present show tubular cristae, as in most alveolates. However, in some gregarines no mitochondria can be observed by utrastructural analysis, while in *Cryptosporidium spp*., only a vestigial mitochondrion can be observed, consistent with the absence of mitochondrial DNA and loss of many of the genes encoding for the mitochondrial electron transport chain (Vaidya & Mather, 2009). In haemosporidia, piroplasms and coccidia, a single mitochondrion can be observed, which may be elongated and branched. In these latter organisms, mitochondrial DNA retains the same three genes (Cox1, Cox2 and CytB) found in dinoflagellate mitochondria, suggesting that loss of the mitochondrial genome occurred only in certain apicomplexan lineages (Vaidya & Mather, 2009).

A similar fate to that of the mitochondrion has been followed by the apicoplast, an organelle thought to be derived from the secondary endosymbiotic event that gave rise to all chromalveolates (Oborník *et al*, 2009). Like mitochondria, apicoplasts seem to have been lost in most gregarines and *Cryptosporidium* spp., but are retained in coccidia, piroplasms and haemosporidians (Xu *et al*, 2004; Toso & Omoto, 2007). The apicoplast is a spherical organelle surrounded by multiple membranes. Although the number of membranes has

been contended (Köhler, 2005), phylogenetic and ultrastructural evidence support the conclusion that the ancestral plastids are comprised of four membranes like those of coccidia, while piroplasms and haemosporidians seem to have lost one of the membranes (Oborník *et al*, 2009). Recently, the closest free-living relative to apicomplexans, *Chromera velia*, was isolated from coral in Australia and shown to have a photosynthetic plastid also comprised of four membranes (Moore *et al*, 2008), further supporting the claim of common ancestry to the chromalveolate plastids. The function of the apicoplast remains an active area of research. Analysis of the genes predicted to traffic to the apicoplast has revealed pathways for the synthesis of fatty acids, isoprenoids and heme (Ralph *et al*, 2004). Consistent with the essentiality of these functions, drugs that target the apicoplast are lethal to apicomplexans, although death is frequently observed a number of cell cycles after treatment in a phenomenon termed delayed death (Ralph *et al*, 2001).

Cytoskeleton

The cytoskeleton of *T. gondii* tachyzoites is organized around three microtubule structures: the conoid, the subpellicular microtubules, and the intraconoidal microtubules (Dubremetz & Ferguson, 2007). By electron microscopy, a number of electron-dense rings are associated with the microtubules and the apical end of the IMC. Two of these rings are associated with apical the end of the conoid, which is an open basket arrangement of a novel tubulin polymer (Figure 2; Hu *et al*, 2002). The conoid sits inside the apical opening of the IMC, and is seen to extrude through the opening during gliding motility and in response to calcium ionophore treatment (as depicted in Figure 2; Mondragón & Frixione, 1996). Immediately bellow the extruded conoid, at the opening of the IMC, another polar ring anchors the 22 subpellicular microtubules that run down two thirds of the length of the parasite. The subpellicular microtubules subtend the IMC, and the pattern and periodicity of intramembrane particles within the IMC suggest an

intimate association between these two structures (Morrissette *et al*, 1997). Finally, the intraconoidal microtubules are a pair of short microtubules that extend from the apical end of the parasite and associate with secretory organelles (Dubremetz & Ferguson, 2007). In extracellular parasites these microtubule structures are not dynamic and are therefore resistant to microtubule disrupting drugs, which are otherwise toxic during parasite replication (Hu *et al*, 2002; Morrissette & Sibley, 2002). Many proteins have been shown to associate with the conoid, as can be expected by the diversity of polymers assembled from the same tubulin subunits (Hu *et al*, 2006).The nature of these proteins is the focus of ongoing research, which will likely illuminate the origin and function of these complex structures.

Microtubule structures are quite diverse among the different apicomplexan zoites. Notably, most haemosporidia, including *Plasmodium spp.*, lack a conoid during most developmental stages except ookinetes, which are the motile zygotes that penetrate the arthropod gut to create the oocyst (Barta, 1989). Although the function of the conoid is a matter of speculation, it has been suggested that the presence of a conoid during the ookinete stage in these species reflects the increased difficulty of penetrating the arthropod gut epithelium, compared to the direct interaction with host cell membranes in the definitive host. If indeed the conoid plays a biophysical role during invasion, the relatively small size of haemosporidian zoites may account for the dispensability of a conoid during these stages. In contrast, polar rings are present in most apicomplexans, as are subpellicular microtubules. The number and arrangement of the latter can vary significantly between different stages and species. In *P. falciparum* these can range from three or four in merozoites to about 60 in ookinetes (Morrissette & Sibley, 2002). Intraconoidal microtubules are absent in many apicomplexans, however it is possible that subpellicular microtubules may take over their function as suggested by their association with micronemes in *P. falciparum* merozoites and sporozoites (Bannister *et al*, 2003; Schrevel *et al*, 2008).

Inner Membrane Complex

As mentioned above, the IMC is formed by vesicles homologous to the alveoli found beneath the plasma membrane of all chromalveolates. In apicomplexans, the vesicles forming the IMC are flattened into plates that closely associated with the plasma membrane, a feature that has been proposed to generate the space necessary for gliding motility to occur (Cavalier-Smith & Chao, 2004). The IMC is composed of a single funnel-shaped plate at the apical end, followed toward the posterior of the cell by rows of rectangular plates, and finally capped at the posterior by a row of triangular plates (Porchet & Torpier, 1977). The plates are thus fused to generate a continuous surface that is only open at the apical and posterior ends. It has been shown by freeze-fracture electron microscopy that intramembrenous particles within the IMC have the same periodicity and pattern as the subpellicular microtubules (Morrissette *et al*, 1997). Given that transmembrane proteins opposite to these particles anchor the motor complex powering motility into the IMC, it is tempting to think that there might be a structural link between the motor and the subpellicular microtubules. Such an interaction could be the molecular key to the directional movement of the parasite, by lending the polarity of the microtubules to the organization of the motors.

While the IMC described above is characteristic of most apicomplexan zoites, the sporozoites of *Theileria* form a notable exception. While *Theileria* has an IMC during its motile ookinete stage within the definitive tick host, other invasive stages lack an IMC (Shaw, 2003). Interestingly, the stages lacking an IMC, like those infecting lymphocytes and erythrocytes of the mammalian intermediate host, enter cells by a zippering mechanism that is independent of parasite actin and distinct from the gliding motility that powers invasion in other apicomplexans (Shaw, 1999). This curious adaptation in *Theileria*, lends weight to the association between the IMC, gliding motility and invasion by most other apicomplexan zoites.

Micronemes

It has been shown in *T. gondii* that proteins from three different organelles are sequentially secreted to mediate invasion: first the micronemes to mediate motility, then the rhoptries to mediate invasion, and finally, once invasion has been completed, the dense granules to remodel the parasitophorous vacuole (Carruthers & Sibley, 1997; Dubremetz *et al*, 1998). This description is almost certainly a generalization, since we now know that proteins from these different compartments interact to mediate invasion (Carruthers & Tomley, 2008), but it serves to illustrate the general pattern of secretion and explain the purpose of the different exocytic organelles. Micronemes are small, rod-shaped organelles that are evenly electron dense and localize to the apical end of the cell (Dubremetz & Ferguson, 2007). They are frequently associated with the cytoskeleton and in *P. falciparum* have been suggested to traffic via the subpellicular microtubules to the apical end (Bannister *et al*, 2003).

The number of micronemes differs significantly between species and developmental stage, with more motile stages bearing greater numbers (Carruthers & Tomley, 2008). This link between micronemes and motility is now well supported in various species by the characterization of adhesins secreted from micronemes and essential for parasite motility. The first microneme proteins were identified as mediating adhesion by *P. falciparum* merozoites (Camus & Hadley, 1985). It was soon realized that, despite differences in their binding affinities, these adhesins belonged to families of proteins found in other *Plasmodium* species and other apicomplexans. However, the first association with gliding motility was reported when the sporozoite adhesin TRAP, which could be knocked out in the asexual stages of *Plasmodium berghei*, was shown to be essential for sporozoite motility and invasion (Sultan *et al*, 1997). The homologue of TRAP in *T. gondii*, MIC2, has also been shown to mediate gliding motility and invasion in a conditional knockout strain (Huynh & Carruthers, 2006). As a whole, microneme proteins vary widely between species,

but similar adhesive domains are frequently observed in diverse arrangements (Tomley & Soldati, 2001). Many microneme proteins are known to reside in complexes with others, and this serves to mediate proper sorting to the micronemes (Huynh & Carruthers, 2006), and presumably expand the repertoire of possible interactions with the host (Carruthers & Tomley, 2008). It has been recently demonstrated that certain microneme proteins serve roles beyond gliding motility. Examples of this in *T. gondii* include MIC8, which is required for invasion but not motility (Kessler *et al*, 2008), and the perforin-like protein PLP1, which has been shown to permeabilize the parasitophorous vacuole prior to egress from the host cell (Kafsack *et al*, 2009).

The mechanism of microneme secretion is not well understood. Secretion is thought to occur at the apical end of the cell through fusion of micronemes with the parasite plasma membrane, although this process has never been observed by electron microscopy. Alternatively, it has been suggested that micronemes are secreted through fusion with rhoptries, although a recent study looking at these organelles found no evidence of this interaction (Lemgruber *et al*, 2010). Micronemes are the only exocytic organelles whose secretion is regulated by intracellular calcium. This was originally demonstrated in *T. gondii*, where chelation of intracellular calcium with BAPTA-AM prevented MIC2 secretion and host cell invasion (Carruthers *et al*, 1999a). It has since been shown to be generalizable to other apicomplexans including *Cryptosporidium* (Chen *et al*, 2004) and *Plasmodium* (Singh *et al*, 2010). Calcium-regulated secretion is common among eukaryotes, and regulates processes from neurotransmitter release (Barclay *et al*, 2005) to the release of defensive proteinaceous spikes in *Paramecium* (Vayssié *et al*, 2000). It is possible that by packaging adhesins into many individual vesicles, oscillations in calcium concentrations allow zoites to release adhesins in bursts over a long period of time, to support gliding motility over longer distances.

Rhoptries

Rhoptries are the other type of secretory organelle associated with the apical end of the parasite. They are elongated and club-shaped, and their narrow necks protrude into the conoid. By freeze-fracture electron microscopy, a number of intramembrane particles can be observed on the cytoplasmic surface of the bulb, aligned in a spiral or a series of parallel rings (Porchet-Hennere & Nicolas, 1983; Lemgruber *et al*, 2010). The contents of rhoptries are electron-dense, but certain staining and sectioning techniques reveal heterogeneity and the presence of lipid membranes and vesicles (Nichols *et al*, 1983; Porchet-Hennere & Nicolas, 1983; Lemgruber *et al*, 2010). Proteins released from the rhoptries have recently been shown to partition into the bulb or the neck, revealing further complexity in the structure of this organelle (Bradley *et al*, 2005).

As with micronemes, the precise site and mechanism of rhoptry secretion remains enigmatic. Electron microscopy studies of invading parasites report the appearance of large electron-lucent vacuoles at the apical end of the parasite, believed to represent recently discharged rhoptries (Nichols *et al*, 1983; Porchet-Hennere & Nicolas, 1983). In a revealing section, the membrane of empty vacuole was observed to be contiguous with the plasma membrane of the parasite and aligned with a small break in the nascent parasitophorous vacuole (Nichols *et al*, 1983). From this observation, it was concluded that rhoptry contents could be secreted directly into the cytoplasm of the host cell during invasion. Although similar observations have not been reported, it is now know that many rhoptry proteins are released into the host cytoplasm (Boothroyd & Dubremetz, 2008). Furthermore, blocking parasite motility with cytochalasin D does not prevent rhoptry release, but induces the formation of small vacuoles called evacuoles, with many of the proteins and characteristics associated with the parasitophorous vacuole (Håkansson *et al*, 2001). It remains unclear whether evacuoles represent the same membranes observed within rhoptries, or newly

formed vacuoles. The answer to this question has important implications for the topology of secreted proteins, and the formation of the parasitophorous vacuole.

A subcellular fractionation followed by mass spectrometry approach has identified many novel rhoptry proteins (Bradley *et al*, 2005). As mentioned above, rhoptry proteins can be broadly divided into rhoptry neck protein (RON) and rhoptry bulb proteins (ROP). To date, ten RONs have been identified, many of them localizing to the moving junction during host cell invasion (Bradley *et al*, 2005; Boothroyd & Dubremetz, 2008; Lamarque *et al*, 2012). RON2 and RON5 are predicted to insert into the host membrane, interact with the host cytoskeleton through RON4 and RON8, and bind the microneme-secreted protein AMA1 on the parasite surface (Figure 3; Lebrun *et al*, 2005; Besteiro *et al*, 2009). Knockout of RON8 demonstrated that formation of this complex is important for efficient invasion (Straub *et al*, 2011). Originally identified in *T. gondii*, formation of this complex at the moving junction has also been reported in *P. falciparum*, suggesting a conserved mechanism for the zoite to introduce its own receptor into the host cell membrane (Alexander *et al*, 2006; Richard *et al*, 2010). Although other mechanisms are clearly important to mediate the interaction between the parasite and host cell, formation of the RON/AMA1 complex may explain the ease with which apicomplexans have adapted to invade such a diversity of host cells.

In contrast to RONs, ROPs are much less conserved and appear to participate in modification of the host cell to prevent cellular immunity and set up an environment suitable for parasite replication (Bradley & Sibley, 2007). In *T. gondii* many ROPs belong to a family of serine/threonine kinases that have been implicated in mouse virulence (Taylor *et al*, 2006; Saeij *et al*, 2006), through the phosphorylation of factors mediating cellular immunity (Jensen *et al*, 2011; Fentress *et al*, 2010). Although no ROPs have been shown to be required for invasion or replication in fibroblasts, the ability of some virulent strains

to survive within activated macrophages has been shown to depend on ROP18 (Fentress *et al*, 2010).

Dense Granules

Compared to rhoptries and micronemes, much less is known about dense granules. By electron microscopy they are spherical, electron-dense structures distributed throughout the cell (Dubremetz & Ferguson, 2007). A number of dense granule proteins (GRAs) have been identified and shown to be secreted into the parasitophorous vacuole following invasion (Carruthers & Sibley, 1997). In *T. gondii* GRA2 and GRA6 are important for generating a tubular network of invaginations from the parasitophorous vacuole membrane (Mercier *et al*, 2002), which is thought to facilitate nutrient exchange with the host-cell cytoplasm. However, in cell culture, knockout of either GRA2 or GRA6 have no growth phenotype in tissue culture, bringing into question the role of these structures. It has recently been shown that a different dense granule protein, GRA15, is important in the modulation of host cell cytokines through the activation of NFκB (Rosowski *et al*, 2011). This observation opens the possibility that, like ROPs, GRAs primarily serve an immune-related function, explaining their dispensable nature for parasites in cell culture.

Gliding Motility and Invasion

Gliding motility is believed to be a general feature of most apicomplexan zoites, although it has only been studied in detail for *T. gondii* and *Plasmodium* species. A number of morphological features appear to be adaptations for this type of motility including a highly polarized cell, apical secretion of micronemes, the IMC, and subpellicular microtubules. All these features are present in motile zoites throughout the phylum with the exception of *Theileria spp.*, which do not perform gliding motility and seem to have developed an altogether different form of invasion. Gliding motility has also been reported in three

unrelated groups of protozoa: diatoms, *Labyrinthula* and *Chlamydomonas*. Although the molecular details of motility in these organisms are not completely understood, ultrastructural features suggest that they represent different types of motility from that found in apicomplexans. In diatoms, gliding occurs through the movement of mucilage deposited in a dedicated slit running across the silica shell of the organism. A thick bundle of actin filaments can be observed running along the same path as the slit, and is thought to provide a track for myosins to move. In contrast to apicomplexan motility therefore, diatoms do not seem to have anchored myosins, and do not rely on *de novo* actin polymerization as demonstrated by their insensitivity to cytochalasin D (CytD) (Heintzelman, 2006). The motility observed in *Labyrinthula*, is also actin based, but unlike apicomplexans, it only occurs within a membrane bound surface generated by the organism, and is therefore akin to the transport of organelles in the cytoplasm (Preston & King, 2005). Finally, gliding motility in *Chlamydomonas* occurs through the transport of protein complexes along the flagellar microtubules and is therefore insensitive to CytD (Kozminski *et al*, 1993). Therefore, the type of gliding motility described below appears to be a unique adaptation of apicomplexans, perhaps as a development of the mechanism still used by colpodellids for myzocytosis (Cavalier-Smith & Chao, 2004).

In apicomplexans, gliding motility consists on the directional translocation of adhesins from their point of secretion at the apical end of the zoite to the posterior of the cell, generating movement in the apical direction. As described above, a number of these adhesins are released from micronemes in response to changes in intracellular calcium concentration (Carruthers & Sibley, 1999). The best-characterized of these adhesins belong to a family of proteins identified by their homology to the *P. falciparum* thrombospondin-related anonymous protein (TRAP; Robson *et al*, 1988), whose homolog in *T. gondii* is MIC2 (Wan *et al*, 1997). Loss of this adhesin has been shown to impair motility in *T. gondii* and *P. berghei* (Sultan *et al*, 1997; Huynh & Carruthers, 2006) demonstrating its

essential role in the process. More recently, careful analysis of gliding motility in *P. berghei* sporozoites lacking TRAP showed movement of adhesive patches without progressive gliding, suggesting that this adhesin may play a special role in motility, which would explain its essentiality in the presence of other microneme adhesins (Münter *et al*, 2009). In *T. gondii*, many of the transmembrane adhesins have been shown to reside in complexes with soluble ones, and the normal function of MIC2 depends on its soluble component M2AP (Huynh *et al*, 2003). Other adhesin complexes have only mild effects on motility in cell culture, and seem to play redundant roles in a process essential for virulence (Cérède *et al*, 2005).

The translocation of adhesins depends on *de novo* actin polymerization. It was first demonstrated in *T. gondii* that actin polymerization in the parasite, not the host cell, is required for gliding motility and invasion, and is therefore sensitive to CytD (Dobrowolski & Sibley, 1996). Regulation of actin polymerization appears critical for normal gliding since most of the cellular actin in *T. gondii* is maintained in the monomeric form and filament stabilizing drugs, like jasplakinolide, lead to aberrant forms of gliding motility (Wetzel *et al*, 2003). Adhesins are tethered to actin filament via aldolase, which has been demonstrated for both MIC2 and TRAP (Jewett & Sibley, 2003; Bosch *et al*, 2007). Translocation of this complex is then achieved via myosin motors anchored in the IMC. The myosin responsible for this movement, MyoA, was identified by its subcellular localization and biophysical properties, and shown to be required for *T. gondii* motility and invasion (Herm-Götz *et al*, 2002; Meissner, 2002). Like other myosins, MyoA is bound by a myosin light chain, MLC1 (known as MTIP in *Plasmodium*) (Herm-Götz *et al*, 2002), and the protein complex in which they are found was dubbed the glideosome, based on its role in gliding (Opitz & Soldati, 2002). Two apicomplexan proteins anchor this complex in the IMC, called glideosome associated proteins (GAP) 45 and 50, according to their molecular weight (Gaskins *et al*, 2004). GAP50 is a transmembrane protein embedded

in the IMC, while GAP45 associates with membranes via N- and C- terminal acylated residues. Recently it has been proposed that GAP45 spans the space within the plasma membrane and the IMC, and a series of elegant experiments suggest that GAP45 length may modulate the distance between the two membranes (Frénal *et al*, 2010). How this array of myosins interacts with short actin filaments to produce directional movement remains an open question. Two possible mechanisms could account for directionality: (*i*.) as proposed above, a link could exist between the glideosome and the subpellicular microtubules, or (*ii*.) a signaling wave could emanate from the apical end, inducing either actin polymerization or motor activity in a directional manner, reminiscent of an axonal action potential. The former possibility is supported by the observation that at least some types of movement during gliding motility seem to follow the same curvature as the subpellicular microtubules (Håkansson *et al*, 1999). All the present evidence indicates that the same mechanism that governs gliding motility also mediates host-cell invasion. Gliding motility during invasion is only distinguished by the adhesin AMA1, which is dispensable for gliding, but necessary for efficient invasion through its interaction with the RON complex at the moving junction (Mital *et al*, 2005; Besteiro *et al*, 2009).

A final aspect of gliding motility is shedding of the adhesins. The first electron microscopic analysis of *P. knowlesii* invasion indicated shedding of a "surface coat" present in extracellular merozoites but absent from the portion of the parasite that had passed the moving junction (Aikawa *et al*, 1978). It is now recognized that this represents shedding of the adhesins from the parasite surface. Although microneme proteins are subject to many proteolytic events (Dowse & Soldati, 2004), processing within the plasma membrane is thought to be the definitive event that removes them from the plasma membrane (Carruthers *et al*, 2000) and this process is essential for efficient invasion (Brossier *et al*, 2003). The proteases responsible for this type of cleavage are called rhomboid proteases (ROMs). The conditional knockout of ROM4 in *T. gondii* leads to the accumulation of adhesins on

the parasite surface, including MIC2 and AMA1, which affects the rate of invasion and gliding motility (Buguliskis *et al*, 2010). While ROM4 is distributed throughout the plasma membrane, ROM1 is localized to the micronemes (Brossier *et al*, 2008) and ROM5 to the posterior of the parasite (Brossier *et al*, 2005). The conditional knockout of ROM1 had only minor effects on parasite replication (Brossier *et al*, 2008), and no knockout of ROM5 has been reported to date. How rhomboids are regulated remains an open question. It seems unreasonable that ROM4 would cleave MIC2 from the surface indiscriminately, whether or not it is being translocated.

ROLE OF CALCIUM IN *T. GONDII* CELLULAR RESPONSES

Calcium is the fifth most abundant element on the earth's crust, present as a divalent cation in practically every environment where life is found. This must have presented a problem for living cells since their origin, because high calcium concentrations lead to protein and nucleic acid aggregation, loss of membrane integrity and phosphate precipitation, all of which are incompatible with life (Case *et al*, 2007). Consequently, calcium efflux pumps and exchangers are found in all organisms that have been analyzed, from prokaryotes to eukaryotes, generating intracellular calcium concentrations thousands of times lower than the environments in which the cells reside (Case *et al*, 2007). It is therefore not surprising that calcium leakage into the cytoplasm is commonly perceived by eukaryotic cells as a signal of membrane rupture (Schapire *et al*, 2009). At the same time, interaction between lipids and calcium induces membrane fusion, suggesting that calcium plays both a signaling and a functional role during membrane repair (Jaiswal, 2001). Fusion of endomembranes with the plasma membrane as a repair mechanism is presumed to be the origin of calcium-regulated exocytosis, which has been adapted for many purposes in eukaryotes. In apicomplexans, calcium-regulated exocytosis governs the release of microneme adhesins, a process central to host-cell invasion (Carruthers & Sibley, 1999). However, the large number of proteins with calcium-binding domains found in apicomplexan genomes suggests that calcium plays a central regulatory function in many biological processes including secretion (Nagamune *et al*, 2008).

Calcium Homeostasis and Signaling in Apicomplexans

Evidence from *Cryptosporidium*, *Plasmodium* and *Toxoplasma* demonstrates that calcium is commonly used as a second messenger in the regulation of apicomplexan host-cell invasion. In all cases the calcium chelator BAPTA-AM, which accumulates in the cytoplasm, abrogated the ability of parasites to secrete adhesins and invade host cells

(Carruthers *et al*, 1999a; Wetzel *et al*, 2005; Chen *et al*, 2004; Singh *et al*, 2010). Like most eukaryotes, *T. gondii* and *Plasmodium spp.* maintain very low levels of intracellular calcium, around 100 nM, in resting cells (Moreno & Zhong, 1996; Garcia *et al*, 1996). However, careful recordings of Fura-2 and Fluo-4 loaded *T. gondii* tachyzoites have shown that calcium concentrations oscillate during motility, are increased by host-cell contact, and are quenched by the initiation of invasion (Vieira & Moreno, 2000; Lovett & Sibley, 2003). Although the natural signals that induce calcium oscillations are not known, artificially increasing intracellular calcium with ionophores has been shown to stimulate motility of intracellular parasites and induce secretion of adhesins (Carruthers *et al*, 1999a; Endo *et al*, 1982).

In mammalian cells, two pathways can contribute to an increase in intracellular calcium. The first occurs in excitable cells like muscle cells, and consists initially of opening calcium channels on the plasma membrane in response to membrane depolarization, followed by amplification of the signal via calcium-induced calcium release from the sarcoplasmic reticulum. The second pathway occurs in non-excitable cells by the activation of a receptor linked to phospholipase C (PLC), which hydrolyses phosphatidylinositol bisphosphate (PIP_2) to generate inositol tiphosphate (IP_3) and diacylglycerol. IP_3 then diffuses to the endoplasmic reticulum (ER) and activates IP_3 receptors that release calcium into the cytoplasm (Moreno *et al*, 2011). Genes encoding many of the components of these pathways have not been identified in apicomplexan genomes. However, it was shown that microneme secretion could be stimulated with ethanol, which is thought to activate PLC (Carruthers *et al*, 1999b), and inhibited by Xestospongin C, which inhibits receptors of the IP_3 family (Lovett *et al*, 2002). Caffeine, an agonist of IP_3 family receptors, was also able to stimulate microneme secretion (Lovett *et al*, 2002). It was also observed that removing extracellular calcium had no significant effect on invasion or gliding motility, suggesting that intracellular calcium stores are both necessary and sufficient for these processes

(Lovett & Sibley, 2003). Taken together, these studies suggest that the mechanism for stimulating microneme secretion via calcium likely involves an IP_3-stimulated calcium channel, and by homology with mammalian models this is probably the ER. Additionally there is pharmacological evidence for ryanodine receptors, which appear to be distinct and perhaps regulating different stores from the IP_3-receptors, given the additive effects of ethanol and ryanodine on calcium concentrations (Lovett & Sibley, 2003). Homologues to mammalian IP_3- and ryanodine-receptors can be found in ciliates (Plattner *et al*, 2012), but none have been identified in apicomplexans, leaving the identity of these channels unresolved. Other stores of intracellular calcium include the mitochondrion, the apicoplast, the acidocalcisomes and the IMC. Both the mitochondrion and the apicoplast lack the channels necessary to be the sources of calcium mobilized during microneme secretion (Moreno *et al*, 2011). Similarly, calcium in the acidocalcisomes is probably bound by polyphosphate, and would require acidification to permit mobilization, a process that does not reflect the rapid kinetics of microneme secretion (Luo *et al*, 2001). Finally, the IMC represents a structure homologous to the alveoli of *Paramecium spp.* which have been shown to be stores of mobilizable calcium (Plattner *et al*, 2012). However, in *T. gondii*, x-ray microanalysis only found significant calcium stores in the acidocalcisomes and ER (Bouchot *et al*, 1999). These observations are therefore consistent with the ER being the source of mobilizable calcium during microneme secretion. Calcium is thought to enter the ER via the sarco/endoplasmic reticulum calcium ATPase (SERCA), for which a homologue has been identified in apicomplexans. Consequently, inhibiting the function of SERCA with thapsigargin can increase calcium concentrations and trigger microneme secretion (Nagamune *et al*, 2007). It is therefore intriguing that thapsigargin and ethanol treatment had an additive effect on calcium concentrations, suggesting the presence of a thapsigargin-insensitive source of calcium (Carruthers *et al*, 1999b). Further work will be needed to identify the calcium channels responding to the different stimuli, in order to

understand what accounts for these observations, and perhaps elucidate the function of calcium oscillations in this process.

Calcium-Binding Proteins and their Functions

The intricate system of calcium release and efflux found in eukaryotes generates temporary surges in cytoplasmic calcium concentrations, which are subsequently transduced into cellular functions by calcium-binding proteins. Broadly speaking, there are two types of protein domains that mediate interactions with calcium: the C2 domain characteristic of protein kinase C (PKC), and the EF domain characteristic of calmodulin (CaM). C2 domains were first described in mammalian PKC as the protein domain responsible for membrane binding in response to calcium, since then they have been found in numerous protein families involved in calcium regulated exocytosis (Barclay *et al*, 2005). Apicomplexans lack an obvious PKC ortholog, although related AGC kinases, like PKG and PKA, are represented in the genome (Peixoto *et al*, 2010). As mentioned above, microneme secretion is thought to occur through the activation of PLC, and this protein has been shown in both mammals and apicomplexans to harbor both C2 and EF domains important for its activity (Fang *et al*, 2006). Other C2 domain containing proteins are poorly characterized in apicomplexans, but a genome search in *T. gondii* reveals more than 20 proteins containing homology to known C2 domains. Recently, a C2 domain containing protein called DOC2 was identified based on a chemically induced, temperature-sensitive *T. gondii* mutant that was resistant to ionophore-induced egress at the non-permissive temperature (Farrell *et al*, 2012). In mammals, DOC2 proteins have been demonstrated to have a positive role in the secretion of growth hormone (Barclay *et al*, 2005). The DOC2 protein in *T. gondii*, and its homolog in *P. falciparum*, appear to play a similar role in mediating the regulated secretion of micronemes (Farrell *et al*, 2012).

The other important calcium-binding domain is the EF domain, which is a helix-loop-helix motif that changes conformation upon calcium binding, frequently exposing hydrophobic surfaces that can mediate binding to other domains or proteins (Lewit-Bentley & Réty, 2000). EF hands are widely distributed among structural and catalytic protein families. In apicomplexans numerous proteins have been found to have either three EF domains like mammalian centrins, or four like mammalian CaMs. Many centrins have been localized to microtubule structures like the spindle and the conoid and are known to be highly dynamic during cell division (Hartmann *et al*, 2006; Hu *et al*, 2006). A single conserved CaM is present in the genomes of most apicomplexans, but CaMs are notably absent from *Cryptosporidium* (Nagamune *et al*, 2008). Myosin light chains (MLCs) are related to CaM, and in mammals are known to play structural and regulatory roles in complex with their cognate myosins. At least seven different MLCs have been identified in *T. gondii*, including the one responsible for anchoring MyoA, MLC1. It is worth noting however, that the EF domains of these proteins are very divergent, frequently lacking the calcium-binding residues, and are therefore thought to function independently of calcium (Polonais *et al*, 2011). A final family of EF-domain containing proteins is the calcium-dependent protein kinases (CDPKs), most of which contain four EF domains (Nagamune *et al*, 2008). In contrast to centrins and CaM, many CDPKs are conserved among apicomplexans and are therefore more likely to play roles in the conserved processes of microneme secretion and invasion.

Calcium-Dependent Protein Kinases of Plants

CDPKs, as their name indicates, are enzymes whose kinase activity is calcium dependent. This dependence is achieved by a domain structure consisting of a kinase domain followed by four calcium-binding EF domains. Current models suggest that calcium binding by the EF-hands changes their conformation thereby activating the kinase domain

(Wernimont *et al*, 2010). In plants, CDPKs have been shown to control a number of cellular processes including metabolism, stress responses, and development (Harper & Harmon, 2005). Their central role in plant biology is demonstrated by their expansion in plant genomes, with over 30 representatives in *Arabidopsis thaliana* (Champion *et al*, 2004). In contrast, the mammalian-type calcium-responsive kinases seem to be under-represented or absent from plant genomes, including PKC-like enzymes and CaM-dependent protein kinases (Harper & Harmon, 2005). The timing and tissue-specific pattern of expression, calcium sensitivity, subcellular localization, and substrate specificity, have all been shown to contribute to the participation of individual CDPKs in different signaling pathways (Harper *et al*, 2004). Many plant CDPKs are known to associate with membranes through N-terminal acylation (Martín & Busconi, 2000).

Most of the cellular pathways regulated by plant CDPKs remain unidentified beyond their participation in broad cellular effects like pollen-tube growth and stress tolerance (Harper *et al*, 2004). Scant evidence linking specific pathways to CDPKs makes it impossible to speculate on any general functions of CDPKs. However, two pathways have been reported that may be of relevance to the regulation of motility in apicomplexans. The first pathway has been shown in maize, where actin-depolymerizing factor is negatively regulated through phosphorylation by CDPKs, suggesting that this pathway may be used to integrate calcium signaling with actin polymerization (Allwood *et al*, 2001). The second pathway concerns the regulation of calcium channels that induce guard cell closing in response to abscisic acid (ABA), which seem to be regulated by two CDPKs, although the targets of phosphorylation remain unknown (Mori *et al*, 2006). As we learn more about the pathways regulated by CDPKs it will be interesting to examine the evolution of CDPKs and their targets to understand how the relationships between kinases and their targets evolve in different organisms.

Calcium-Dependent Protein Kinases of Apicomplexans

A phylogeny of the EF domain-containing kinases of *T. gondii*, *P. falciparum* and *Cryptosporidium parvum* reveals five groups of kinases conserved among all three species (Billker *et al*, 2009). Four of these groups have the canonical CDPK domain arrangement with a kinase domain followed by four EF domains, while the fifth contains additional EF domains in the N-terminus, and a variable number of C-terminal domains. In *T. gondii*, one of the conserved canonical CDPKs is duplicated, and six other EF-containing kinases are found: one canonical and conserved with *C. parvum*, one non-canonical and conserved with *P. falciparum*, and four non-canonical and absent from the other genomes (Figure 4; Billker *et al*, 2009). Non-canonical CDPKs are also present in plants, although the role of calcium in their regulation has not been investigated (Harper *et al*, 2004). As in plants, many apicomplexan CDPKs are predicted to be N-terminally acylated, and the putative acylation sites are frequently conserved across the different species examined (Billker *et al*, 2009). It is possible that subcellular localization will play a role in the function of these kinases.

In *P. berghei*, many of the CDPKs have been shown to be dispensable for the asexual cycle in erythrocytes. Knockout of these CDPKs has revealed defects at various developmental stages following the asexual cycle including male gamete exflagellation (PbCDPK4), ookinete motility (PbCDPK3), and sporozoite invasion of hepatocytes (PbCDPK6) (Billker *et al*, 2004; Coppi *et al*, 2007; Siden-Kiamos *et al*, 2006). However, none of the cellular pathways regulated by these kinases have been identified, and developmental defects preclude the examination of later developmental stages. More recently, tagging endogenous *P. falciparum* CDPK5 with a destabilization domain allowed investigators to regulate its degradation, and demonstrate a role for PfCDPK5 in merozoite egress from erythrocytes (Dvorin *et al*, 2010). Surprisingly, mechanical disruption liberated the merozoites and allowed them to infect new erythrocytes, suggesting that the defect is

restricted to egress and not invasion. Using inhibitors to PfCDPK1, a different study showed a block between schizogony and invasion, although such a defect could be caused by non-specific inhibition of other kinases (Kato *et al*, 2008). Together, these studies suggest a link between CDPKs and motility in *P. falciparum* merozoites.

Pharmacological studies suggest that *Plasmodium knowlesi* and *T. gondii* invasion are blocked by broad-spectrum serine/threonine kinase inhibitors acting downstream of calcium fluxes (Ward *et al*, 1994; Carruthers *et al*, 1999a). In *T. gondii*, the presumed target of one such kinase inhibitor, KT5926, was suggested to be TgCDPK1, the second most highly conserved CDPK among apicomplexans (Kieschnick *et al*, 2001). However, as they lack genetic evidence, these studies are at best suggestive of a role for TgCDPK1 in *T. gondii* invasion and motility. Unfortunately, new preparations of KT5926 lack the activity previously identified, suggesting that previous effects might have been caused by contaminants (C. Beckers, personal communication). Furthermore, it is possible that these inhibitors have additional targets that account for their effects *in vivo*, such as other CDPKs. However this pharmacological evidence, along with the known activation of CDPKs by calcium, has encouraged research into the possible roles of these kinases in calcium-regulated secretion and invasion. Understanding the functions of CDPKs in apicomplexans will illuminate the central role of calcium regulation in gliding motility, and potentially uncover new cellular pathways participating in this essential process.

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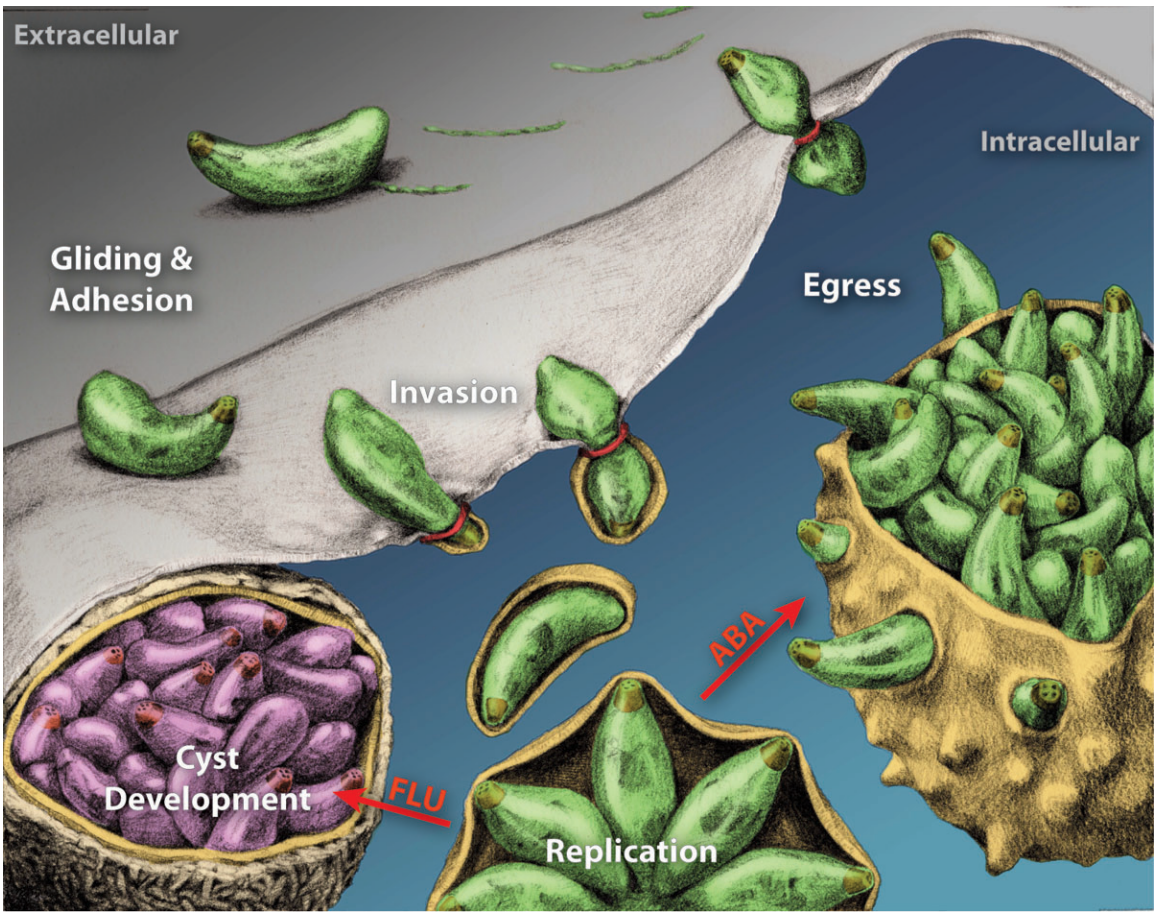

Figure 1. Intracellular cycle of *T. gondii*. Tachyzoites display gliding motility on the substratum and along the surface of cells and use this unique form of actin-myosin based motility to actively invade the host cell. Attachment is mediated by secretion of microneme proteins from the apex of the parasite marked by the conical cap known as the conoid. At the point of invasion, a tight constriction occurs between the host and parasite membranes, formed by a ring of proteins secreted from the rhoptries (depicted as a red ring). Intracellular replication leads to enlargement of the vacuole and accumulation of abscisic acid (ABA), which eventually triggers egress. Blocking production of ABA with the plant herbicide fluoridone (FLU) prevents egress and leads to development of semidormant cysts. Changes in cytosolic calcium have been shown to oscillate during gliding motility, control microneme secretion, and activate egress, as described in the text. [Adapted from (Billker *et al*, 2009); reproduced with permission of Elsevier]

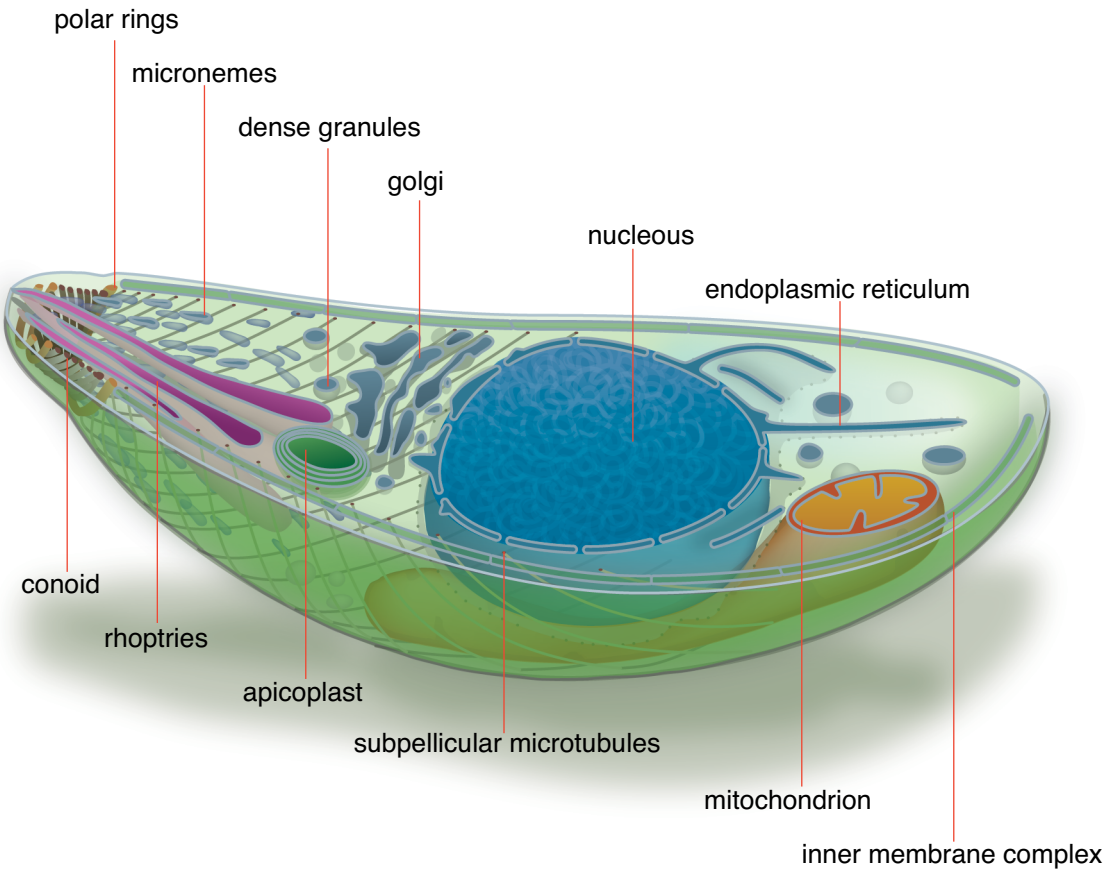

Figure 2. Cross-section of a *T. gondii* tachyzoite. Diagram depicts an extracellular tachyzoite with the conoid extruded. For clarity, structures are not drawn to scale, and secretory organelles are less abundant that in actuality. Representative structures are labeled.

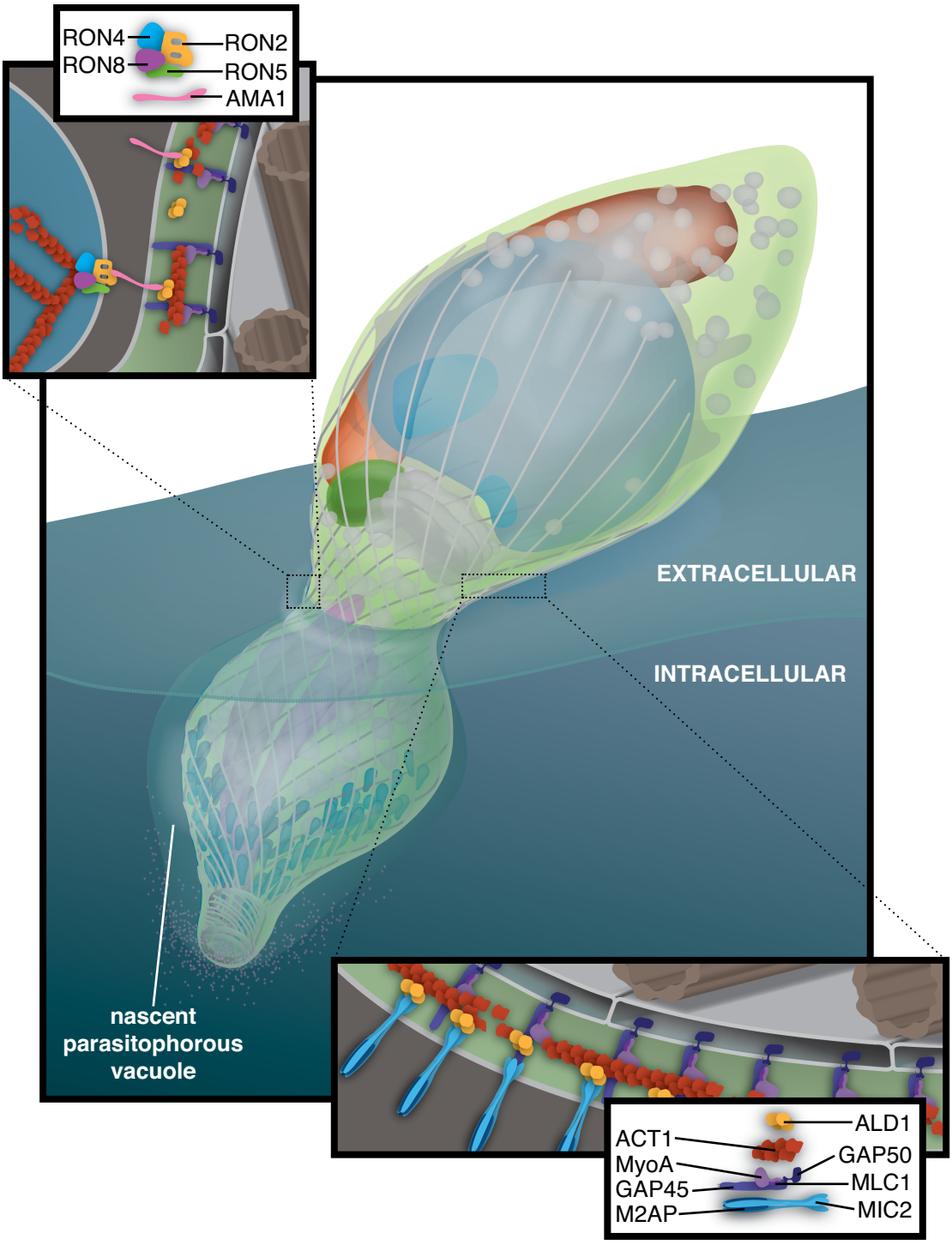

Figure 3. Invasion of host cells by *T. gondii*. Central diagram depicts a *T. gondii* tachyzoite invading a host cell. Insets illustrate the protein interactions that mediate moving junction formation (top left) and gliding motility (bottom right).

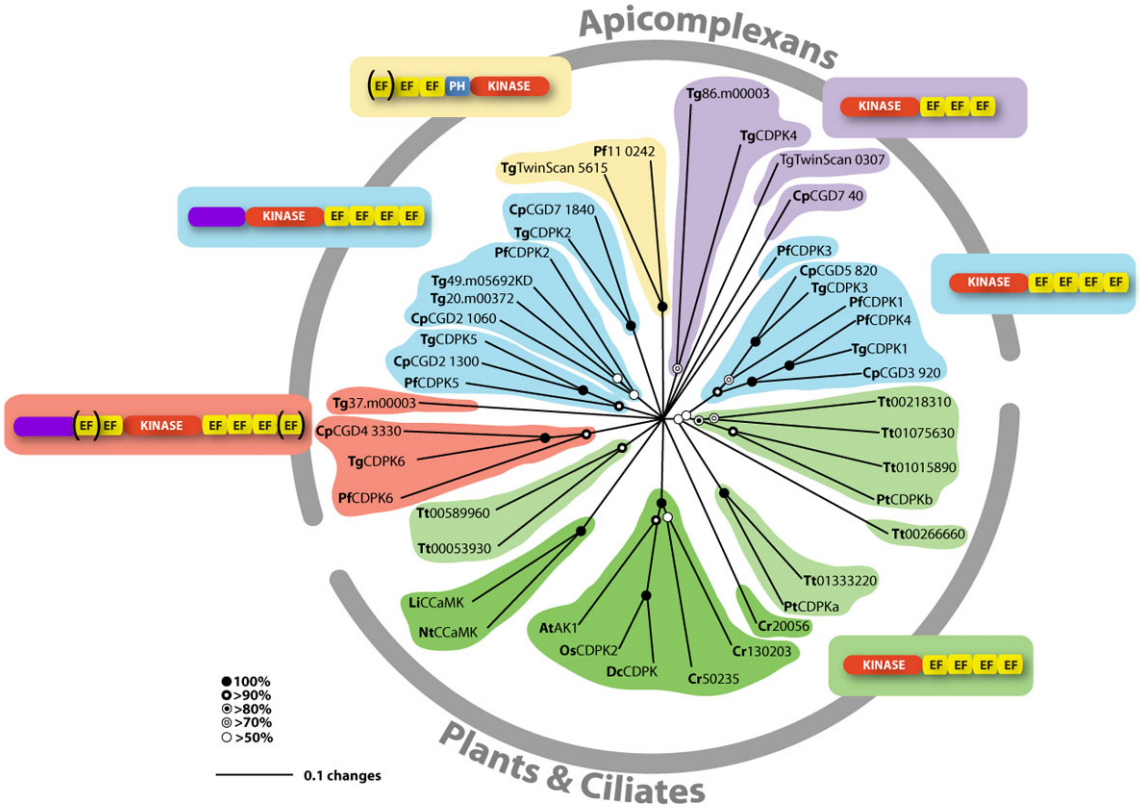

Figure 4. Phylogenetic analyses of calcium-dependent kinases in apicomplexans and plants. Comparison of calcium-dependent protein kinases in higher plants (green), ciliates (light green), and apicomplexans. The canonical domain architecture is represented by plant CDPKs consisting of an N-terminal kinase domain followed by four EF hand domains (EF). Two groups of apicompelxan CDPKs fit this canonical profile (blue). One group has relatively short N-terminal regions (right side), while most members of the other have an extended N terminus that is not homologous to other known domains (left side). Alternative domain structures are shown for other apicomplexan CDPKs, including a group with only three C-terminal EF hand domains (purple), a group with two or three N-terminal EF hand domains followed by a plekstrin homology (PH) and kinase domains (yellow), a group with one or more N-terminal EF hand domains, a kinase domain, and three or four C-terminal EF hand domains (red). Representative models are shown; () indicate variable number of domains. Gene names are based on previously published names or based on orthologous groupings defined here. Phylogenetic tree drawn using a distance matrix based on comparison of the kinase domains using Neighbor joining, BioNJ algorithym in PAUP* (Swofford, 1991); bootstrap values shown in filled circles (n = 100). Taxa: Tg, *T. gondii*; Pf, *P. falciparum*; Cp, *C. parvum*; Tt, *Tetrahymena thermophila*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Dc, *Daucuscarota*; Cr, *Chlamydomonas reinhardtii*; Pt, *Paramecium tetraurelia*; Li, *Lilium longiflorum*; Nt, *Nicotiana tabacum*. [Adapted from (Billker *et al*, 2009); reproduced with permission of Elsevier]

CHAPTER II

A Calcium-Dependent Protein Kinase is an Essential Regulator of Exocytosis in *Toxoplasma gondii*

PREFACE

Work presented in this chapter was conducted by Sebastian Lourido and Joel Schuman. SL designed and performed the majority of experiments, analyzed the data, and generated the figures. JS performed the video miscopy measurements of motility and analyzed the data. Raymond Hui provided key insight into the regulation of CDPKs by calcium. Kevan M. Shokat and Chao Zhang provided inhibitors and insight into the strategy for chemical biology experiments. L. David Sibley supervised the project and assisted with experimental design and analyses. The first draft of this chapter was written by SL. Comments from LDS were incorporated into the final version presented here. This chapter was published in its entirety and is reproduced here with permission of Nature Publishing Group [Lourido S, Shuman J, Zhang C, Shokat KM, Hui R & Sibley LD (2010) Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in Toxoplasma. Nature 465: 359–362]

MANUSCRIPT

Calcium-regulated exocytosis is a ubiquitous process in eukaryotes, whereby intracellular vesicles synchronously fuse with the plasma membrane in response to an intracellular calcium surge (Barclay et al, 2005). This process regulates diverse cellular functions like plasma membrane repair in plants and animals (Bansal & Campbell, 2004; Schapire et al, 2009), discharge of defensive spikes in *Paramecium* (Vayssie et al, 2000), and secretion of insulin from pancreatic cells, immune modulators from lymphocytes, and chemical transmitters from neurons (Chieregatti & Meldolesi, 2005). In animal cells, serine/threonine kinases including PKA, PKC and CAM-kinases have been implicated in the calcium-signal transduction leading to regulated secretion (Barclay et al, 2005; Choi et al, 2002; Easom, 1999). Although plants and protozoa also regulate secretion via intracellular calcium, the means by which these signals are relayed have not been elucidated. Here we demonstrate that the *Toxoplasma gondii* calcium-dependent protein kinase 1 (TgCDPK1) is an essential regulator of calcium-dependent exocytosis in this opportunistic human pathogen. We present genetic evidence that TgCDPK1 controls calcium-dependent secretion of specialized organelles called micronemes. The disruption of microneme secretion was manifested as a block of essential phenotypes including parasite motility, host-cell invasion, and egress. We further demonstrate that pyrazolopyrimidine-derived compounds specifically inhibit TgCDPK1, disrupting the parasite life cycle at stages dependent on microneme secretion. Inhibition was specific to TgCDPK1, since expression of a resistant kinase allele reversed the sensitivity of *T. gondii* to the inhibitor. Our results demonstrate that TgCDPK1 is an essential regulator of calcium-dependent secretion in *T. gondii*. TgCDPK1 is conserved among apicomplexans and belongs to a family of kinases shared with plants and ciliates (Billker et al, 2009), suggesting that it may play a role in calcium-regulated secretion in other organisms. Since this kinase family is absent from

mammalian hosts, it represents a validated target that may be exploitable for chemotherapy against *T. gondii* and related apicomplexans.

The apicomplexan parasite *T. gondii* has been used as a model to describe the secretion of numerous proteins from specialized organelles, called micronemes, in response to increased intracellular calcium (Carruthers & Sibley, 1999). Microneme secretion can be blocked by serine/threonine kinase inhibitors, and this is not circumvented by manipulation of intracellular calcium levels using calcium ionophores, suggesting kinases mediate the transduction of the calcium signal (Carruthers et al, 1999a). Calcium-dependent protein kinases (CDPKs), have been identified in plants, ciliates, and apicomplexans, but are absent in fungi and animals (Harper & Harmon, 2005). CDPKs are characterized by a kinase domain fused to a calcium-binding, calmodulin-like domain (Harper & Harmon, 2005). The calmodulin-like domain holds the kinase domain in an inactive conformation, releasing inhibition in the presence of calcium. Recent structural studies illustrate a novel mechanism of CDPK activation that results from a large-scale intramolecular rearrangement (Wernimont et al, 2010). Apicomplexans contain a diverse family of CDPKs, some of which have canonical domain structures, while others are more diverse (Billker et al, 2009). In *Plasmodium*, the causative agent of malaria, knockouts in several of these CDPKs result in blocks at different developmental stages, although no link to specific cellular processes has been established (Billker et al, 2009). Chemical inhibition using KT2936, a compound related to the pan-kinase inhibitor staurosporine, has been shown to inhibit *T. gondii* binding to host cells (Kieschnick et al, 2001). Although this compound inhibits TgCDPK1 *in vitro*, it is unlikely to provide specific inhibition of this target in the parasite or the host, and the cellular pathways it affects remain unknown (Kieschnick et al, 2001).

To define the role of TgCDPK1 in the parasite life-cycle, we generated a conditional knockout (cKO) using the tetracycline trans-activator system, previously developed for the study of essential genes in *Toxoplasma* (Meissner et al, 2002). We engineered strains

expressing a HA9-tagged allele of TgCDPK1 driven by the *SAG1* minimal promoter preceded by a series of seven tetracycline-operator sequences (i.e. *TetO7SAG1*), permitting repression of the transgene during growth in anhydrotetracycline (ATc; Figure 1A). The endogenous *TgCDPK1* gene was then replaced with a drug resistance marker by double homologous recombination, generating the cKO (Figure 1A). The presence of the endogenous and/or regulatable alleles was assessed by polymerase chain reaction, using primers against consecutive exons and the intervening intron (Figure 1B). Different alleles, expressed under the *SAG1* constitutive promoter, were subsequently introduced into the cKO to test for complementation. Growth of the cKO for 72h in the presence of ATc resulted in nearly undetectable levels of the HA9-tagged regulatable protein, while the c-Myc-tagged constitutive proteins were stably expressed in complemented strains grown in the presence of the drug (Figures 1C and 1D).

In fibroblast monolayers, *Toxoplasma* has the ability to replicate, lyse and reinfect neighboring cells to produce a zone of clearance, or plaque, which can be used as an initial measure of fitness. As a first assessment of the contribution of TgCDPK1 to the parasite life cycle, we tested the ability of different parasite clones to form plaques over the course of seven days. Wild type parasites (WT) formed plaques equally in the presence or absence of ATc, confirming its lack of toxicity to parasites, as previously observed (Meissner et al, 2002). The cKO also grew normally in the absence of ATc, however the presence of the drug led to a complete block in plaque formation (Figure 1E). The phenotype was fully rescued in strains complemented with the wild type *allele* (cKO/*WT*; Figure 1E). Complementation depended on the kinase activity of TgCDPK1, since an allele where the catalytic aspartate in the kinase domain was mutated to an alanine was unable to rescue the cKO (cKO/$D^{174}A$; Figure 1E).

Motility in apicomplexan parasites depends on a unique system whereby adhesins contained in the micronemes are released onto the apical end of the parasite and translocated

to the posterior of the cell by a protein complex termed the glideosome, thus propelling the parasite forward (Sibley, 2004; Soldati & Meissner, 2004). Closer analysis of the cKO strain showed that after 72h of growth in the presence of ATc, despite replication to wild type levels, parasites were significantly impaired in all forms of gliding motility (Figure 2A). Surprisingly, those cKO parasites that did glide exhibited wild type speeds of motility, suggesting that the rate of adhesin translocation remained unaffected (Supplementary Figure 1). To study whether the inhibition of gliding motility corresponded to a defect in invasion, we incubated equal numbers of parasites with fibroblast monolayers for 30 min and washed off unbound parasites before fixing the cells. Intracellular and extracellular parasites were distinguished by differential staining, and counted by fluorescence microscopy. The cKO experienced greater than 90% reduction in invasion when grown in the presence of ATc (Figure 2B). Just as with plaque formation, invasion could be rescued by expression of the constitutive *WT* allele, but not the kinase dead allele (cKO/*WT* and cKO/$D^{174}A$, respectively; Figure 2B). Interestingly, the lack of invasion did not result in an increased number of extracellular parasites adhered to the monolayer, in contrast to what has been observed for mutation or inhibition of actin polymerization or motor activity (Sibley, 2004; Soldati & Meissner, 2004).

Egress from host cells has been shown to depend on many of the same cellular pathways required for invasion (Kafsack et al, 2009; Nagamune et al, 2008). During natural egress, accumulation of the hormone abscisic acid causes an increase in parasite cytoplasmic calcium, leading to the activation of secretion and motility (Nagamune et al, 2008). Experimentally, egress can be more effectively triggered by artificially increasing cytoplasmic calcium with ionophores (Endo et al, 1982). The cKO parasites grown in the absence of ATc, behaved like WT and rapidly egressed from host cells in response to ionophore treatment (Figure 2C). In contrast, cKO parasites grown in the presence of ATc, did not respond to ionophore, remaining immotile for up to six min after addition of

ionophore (Figure 2C). Together these experiments suggest that TgCDPK1 is essential for the transduction of the calcium signals regulating gliding motility, invasion, and egress.

Based on the above experiments, it was evident that all of the phenotypes depending on TgCDPK1 shared a requirement for adhesins stored in micronemes, which undergo calcium-regulated exocytosis (Carruthers & Sibley, 1999). Interestingly, TgCDPK1 is among the 100 genes with a similar expression pattern to known microneme proteins, as detected by microarray analysis of synchronized parasites (95% CI; M. Behnke and M. White unpublished data). Together, these data suggested the possibility that TgCDPK1 regulates microneme secretion, releasing, among other proteins, the well-studied adhesin MIC2 (Carruthers & Sibley, 1999). Following secretion onto the cell surface, MIC2 is translocated to the cell posterior and shed from the parasite surface by proteolysis, allowing for the detection of secreted MIC2 in the supernatant (Carruthers et al, 2000). As expected, MIC2 was detected in the supernatant of WT parasites stimulated with ethanol, which is another potent secretagogue that is thought to act through phospholipase C (Carruthers et al, 1999b) (Figure 3A). In contrast, the amount of MIC2 secreted by the cKO parasites grown in the presence of ATc was nearly undetectable, demonstrating a severe defect in calcium-regulated exocytosis (Figure 3A). Secretion of MIC2 was restored to wild type levels by the constitutive *WT* allele, but not by the kinase dead allele (Figure 3A). In contrast, the constitutively secreted, dense-granule protein, GRA1, was detected in the supernatant of all strains, and its levels were unaltered by ATc treatment, demonstrating that TgCDPK1 specifically regulates calcium-dependent exocytosis (Figure 3A).

Microneme secretion is also thought to play an important role in parasite egress. Consistent with this, release of a perforin-like protein (TgPLP1) from the micronemes was shown to play an important role in the permeabilization of the parasitophorous vacuole membrane (PVM) during natural egress (Kafsack et al, 2009). As an independent method for assessing microneme secretion at this crucial late stage of infection, we generated WT

and TgCDPK1 cKO lines expressing a constitutively secreted form of DsRed, allowing for the visualization of PVM integrity by live video microscopy. To avoid premature rupture of the vacuole by mechanical stress induced by motility, we treated parasite with cytochalasin D, in order to immobilize them. In treated cells, we were able to selectively monitor the kinetics of perforin-mediated PVM rupture by leakage of DsRed from the vacuole. As previously reported, WT parasites rapidly permeabilized the PVM upon calcium-ionophore treatment, releasing DsRed into the host-cell cytoplasm (Figure 3B). All WT vacuoles observed showed rapid PVM permeabilization (average 1.7 ± 0.5 min, s.d.), whereas approximately 30% of cKO parasites grown in the presence of ATc failed to rupture the PVM during the 10-minute observation period (Figure 3C; data not shown). Those cKO parasite vacuoles that did release DsRed, showed a significant delay compared to WT vacuoles (average 4.8 ± 1.3 min SD; Figure 3C). Additionally the rate at which DsRed was lost from the vacuole of cKO parasites was significantly slower compared to WT (Figure 3D). Collectively, these results demonstrate a requirement for TgCDPK1 in controlling the release of microneme contents including TgPLP1 during egress. Moreover, the inability of calcium-ionophore to circumvent the requirement for TgCDPK1, places this kinase as a critical transducer of the calcium signal regulating microneme exocytosis.

Having established the crucial role of TgCDPK1 in several essential phenotypes, we sought to determine whether direct chemical inhibition of this kinase could have the same devastating effect on the parasite life cycle. To do this, we took advantage of the atypical nucleotide-binding pocket of TgCDPK1. It has been previously reported that mutation of a specific residue, termed the gatekeeper residue, can change a kinase's affinity for inhibitors of the pyrazolopyrimidine class (PP1) (Bishop et al, 2000; Burkard et al, 2007). While most kinases in both the mammalian and parasite genomes harbor bulky residues at the gatekeeper position, TgCDPK1 is unusual in that it displays a glycine. This glycine gatekeeper is unique among canonical CDPKs (Figure 4A) and in fact all protein

kinases predicted to be active in *T. gondii* (L. Peixoto and D. Roos, unpublished data). This finding predicts that wild type TgCDPK1 will be sensitive to PP1-based inhibitors like 1-tert-Butyl-3-(3-methyl-benzyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (3-MB-PP1; Figure 4B) (Burkard et al, 2007), while mutation of the gatekeeper to a bulkier residue like methionine should render the kinase resistant. To test this prediction, the TgCDPK1 cKO was complemented with either wild type or gatekeeper-mutant kinase alleles (cKO/*WT* and cKO/$G^{128}M$, respectively), cloned under the TgCDPK1 endogenous promoter to ensure levels of expression comparable to the endogenous kinase. Both alleles restored plaque formation in the cKO lines, demonstrating that the mutation of the TgCDPK1 gatekeeper residue to a methionine has no major deleterious effects on its function (Supplementary Figure 2). As an initial measure of the sensitivity of *WT* TgCDPK1 to 3-MB-PP1, we treated parasites with drug or dimethyl sulfoxide (DMSO) vehicle, prior to testing their ability to invade host cells. Both WT and cKO/*WT* parasites showed a significant decrease in invasion in the presence of 3-MB-PP1, while neither showed an increase in extracellular attachment (Figure 4C), similar to the cKO phenotype in the presence of ATc (Figure 2B). In contrast, cKO/$G^{128}M$ showed no loss in invasiveness upon 3-MB-PP1 treatment, demonstrating the drugs effects on invasion are caused by the specific inhibition of TgCDPK1. A similar result was observed for egress, where ionophore treatment failed to induce drug-treated cKO/*WT*, while cKO/$G^{128}M$ egressed normally (Supplementary Figure 3). The phenotypes in invasion and egress are directly attributed to the inhibition of calcium-dependent microneme secretion, because treatment with 3-MB-PP1 inhibited MIC2 secretion in both WT and cKO/*WT* strains, while having negligible effects on cKO/$G^{128}M$ (Figure 4D). Together these data demonstrate that chemical inhibition of TgCDPK1 phenocopies the genetic repression and blocks the parasite life cycle at multiple microneme-dependent stages, preventing egress of parasites from infected cells and host cell infection.

Our findings demonstrate that TgCDPK1 acts as an essential regulator of calcium-dependent exocytosis in *T. gondii*, functioning downstream of the calcium surge. This crucial function can be successfully inhibited using simple small molecules that lack significant host toxicity, serving as proof-of-principle that specific inhibition of this essential kinase has the potential to block functions vital to the parasite. TgCDPK1 is conserved in other apicomplexans, where it may have a similar function, and also serve as a potential drug target, particularly since this class of kinases is absent from mammalian hosts, thus minimizing off-target effects and toxicity. Although PP1 analogues have been exploited to selectively inhibit mutated mammalian kinases (i.e. altered to contain a small gatekeeper) (Bishop et al, 2000; Burkard et al, 2007), they normally show limited sensitivity to this class of inhibitors, making them potential lead compounds for anti-parasitic drugs. More broadly, this work opens the possibility that CDPKs may regulate calcium-dependent exocytosis in other organisms like ciliates and plants, representing an evolutionary precedent to calmodulin-dependent kinases that regulate exocytosis in animals. The flexibility of *T. gondii* as a model system may thus prove useful for probing additional features of calcium regulated secretion that are conserved in eukaryotic cells.

METHODS SUMMARY

Growth of Host Cells and Parasite Strains

T. gondii tachyzoites were maintained by growth in monolayers of human foreskin fibroblasts (HFFs) cultured as previously described(Starnes et al, 2009). Growth media for complemented strains was supplemented with 3 M pyramethamine (Sigma). Where noted, ATc (Clontech) was added at a concentration of 1.5 g/ml and treatment was typically done for 72h.

Cellular Assays

Plaque formation and invasion assays were performed as previously described(Huynh et al, 2003; Roos et al, 1994). For invasion 5 x 10^6 parasites in a 250 l volume were added to sub-confluent HFF monolayers and allowed to invade for 20 min. For inhibitor studies, parasites were incubated in 5 M 3-MB-PP1 (synthesized by the laboratory of KMS) or vehicle-only control (DMSO), for 20 min at room temperature, prior to invasion.

Secretion

Microneme secretion was assayed as previously described(Carruthers et al, 1999b) by monitoring the release of MIC2 into the culture medium. Secretion was stimulated by treatment with 3%FBS and 2% ethanol, 15 min at 37 C. Samples were resolved by SDS-PAGE, blotted and probed with mouse-α-MIC2 (mAb 6D10), rabbit-α-TgACT1, and mouse-α-GRA1 (mAb Tg17-43). A FLA-5000 phosphoimager (Fuji) was used for quantitation. For inhibitor studies, parasites were pre-treated with 5 M 3-MB-PP1 or vehicle-only control (DMSO) for 20 min at 37 C, prior to stimulation.

Egress and Vacuole Permeabilization

Egress and PVM permeabilization were monitored by video microscopy. When noted, parasites were pre-incubated for 10 min in culture media containing 2 M Cytochalasin D (Calbiochem) at 37 C. Egress was induced with 8 M calcium-ionophore A23187 (Calbiochem), and vacuoles were imaged for up to 10 min following its addition. To quantify permeabilization, fluorescence within an 80 m^2 region of each vacuole was measured using Openlab v. 4.1 (Improvision). The values for each vacuole were, normalized against the starting (100%) and ending (0%) values for that particular vacuole, and the derivative of the curve was used to find the maximal rate of fluorescence loss and the time when that rate occurred.

METHODS

Growth of Host Cells and Parasite Strains

T. gondii tachyzoites were maintained by growth in monolayers of human foreskin fibroblasts (HFFs) cultured in Dulbecco's modified Eagle's medium containing 10% tetracycline-free fetal bovine serum (HyClone) , 2mM glutamine, 10mM HEPES (pH 7.5), and 20 g/ml gentamicin. Chloramphenicol (20 g/ml; Sigma), phleomycin (5 g/ml; InvivoGen), ATc (1.5 g/ml; Clontech), and pyrimethamine (3 M; Sigma) were added to the media during selection as indicated, and for the maintenance of merodiploid or complemented strains. When noted, ATc was used at a concentration of 1.5 g/ml and parasites were treated for 72 h.

Strain Construction

The TgCDPK1 cKO was constructed using tetracycline transactivator system(Meissner et al, 2002), as previously described for TgALD1(Starnes et al, 2009). Briefly, *TgCDPK1* (Genbank accession number AF333958) was cloned with a C-terminal HA9-tag into the pTetO7SAG1 vector (obtained from Dominique Soldati), downstream of the inducible promoter, and the *CAT* selectable marker driven by the *SAG1* promoter was introduced at a different site. The TATi-1 strain (obtained from Dominique Soldati), used as the wild type (WT) background in this study, was transfected with the regulatable construct, and stable merodiploids were selected with chloramphenicol (Kim et al, 1993), and cloned by limiting dilution. To generate the knockout construct the *Ble* selectable marker (Messina et al, 1995) was flanked with 1.5kb upstream of the *TgCDPK1* start codon and 1.5kb downstream of the stop codon, followed by a YFP expression cassette (Starnes et al, 2009). The knockout construct was linearized and transfected into the merodiploid strain and stable pools were selected through two rounds of phleomycin selection (Messina et al, 1995). Sorting for YFP-negative parasites was used to enrich for

successful knockouts and individual clones were isolated by limiting dilution. Knockout of the endogenous *TgCDPK1* gene was confirmed in clones by polymerase chain reaction, using primers against consecutive exons and the intervening intron, to distinguish between the endogenous and regulatable alleles. Complementing plasmids were constructed by cloning *TgCDPK1* with a carboxyl-terminal c-Myc-tag, under the regulation of the *SAG1* promoter. The *DHFR* selectable marker conferring pyramethamine resistance (Donald & Roos, 1993) was cloned into the complementing vectors. For the inhibitor studies, *SAG1* was replaced with the 1.5kb region preceding the *TgCDPK1* start codon. Co-transfection with pDHFR-TS (Donald & Roos, 1993) was used to generate stable clones. Mutations were generated by site directed mutagenesis. Complementing plasmids were transfected into the cKO, stable lines were selected with pyramethamine, and clones were isolated by limiting dilution. To monitor PVM permeabilization, WT and cKO strains were transfected with p30-DsRed (Kafsack et al, 2009) (obtained from Florence Dzierszinski) and pDHFR-TS for isolation of stable transgenic lines as described above.

Plaque Assay

Plaque assays were performed as previously described(Roos et al, 1994). Confluent monolayers of human foreskin fibroblasts in 6-well plates were infected with 300 parasites per well in media with or without 1.5 g/ml ATc (Clontech). 24 h post infection additional media was added reduce the concentration of ATc to 1 g/ml. Monolayers were fixed 7 days post infection and stained with crystal violet. Experiments were repeated three times with triplicate wells / experiment.

Invasion Assays

Parasites were harvested in invasion media (Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.5, supplemented with 3% FBS). 5 x 10^6 parasites in a

250 l volume were added to sub-confluent HFF monolayers and allowed to invade for 20 min. Monolayers were then fixed and stained as previously described (Huynh et al, 2003) to distinguish extracellular from total parasites. Three experimental replicates were performed for each strain in each of three separate experiments and parasite numbers per field were normalized to host-cell nuclei. For the inhibitor studies, parasites were incubated in 5 M 3-MB-PP1 or vehicle-only control (DMSO), for 20 min at room temperature, prior to invasion.

Immunofluorescence Microscopy

Intracellular parasites were stained as previously described (Starnes et al, 2009). MIC2 staining within the micronemes required a 2 min permeabilization with cold 100% ethanol on ice. Staining was performed with rabbit-α-HA9 (Invitrogen) and mouse-α-MIC2 (mAb 6D10), followed by Alexa564-goat-α-rabbit IgG (Invitrogen), Cy5-goat-α-mouse IgG (Jackson) and Sytox green (Invitrogen) for the nuclear stain. Images were collected on a Zeiss LSM 510 confocal microscope.

Video Microscopy and Quantitation of Gliding Motility

Gliding and egress were analyzed by video microscopy as previously described(Håkansson et al). For gliding, 75 images were taken with exposure times ranging from 50-100 milliseconds with 1 second between exposures. Images were collected and combined into composites using Openlab v. 4.1 (Improvision). ImageJ was used to analyze the images. The ParticleTracker plug-in (Sbalzarini & Koumoutsakos, 2005) was used to track cell motility and Cell Counter was used to quantify percent motility.

Ionophore-Induced Egress and PVM Permeabilization

Egress and PVM permeabilization were monitored by video microscopy as described above. Where noted, parasites were pre-incubated for 10 min in media containing 2μM Cytochalasin D (Calbiochem) at 37°C. All dishes were allowed to equilibrate for 5 min on the heated stage, prior to the addition of 8μM calcium ionophore A23187 (Calbiochem). Vacuoles were imaged for up to 10 min after the addition of ionophore. To quantify vacuole permeabilization the fluorescence intensity within a 80μm2 region of each vacuole was measured using Openlab. The values for each vacuole were normalized against the starting (100%) and ending (0%) values for that particular vacuole, and the derivative of the curve was used to find the maximal rate of fluorescence loss and the time when that rate occurred. For the inhibitor studies, parasites were pre-treated with 5μM 3-MB-PP1 or vehicle-only control (DMSO) for 20 min at 37°C, prior to the addition of ionophore.

MIC2 Secretion Assay

Microneme secretion was assayed as previously described(Carruthers et al, 1999b) by monitoring the release of MIC2 into the culture medium. Secretion was stimulated by treatment with 3%FBS and 2% ethanol, 15 min at 37 C. Parasite lysis was monitored by the release of actin into the medium and remained undetectable in all experiments presented. GRA1 secretion was used as a control for constitutive secretion. Samples were resolved by SDS-PAGE, blotted and probed with mouse-α-MIC2 (mAb 6D10), rabbit-α-TgACT1, and mouse-α-GRA1 (mAb Tg17-43, kindly provided by Marie France Cesbron, Genoble, France). Quantitation was performed by densitometry using a FLA-5000 phosphoimager (Fuji Medical Systems). For the inhibitor studies, parasites were pre-treated with 5 M 3-MB-PP1 or vehicle-only control (DMSO) for 20 min at 37 C, prior to stimulation.

Statistics

Experiments were repeated three or more times and statistical analyses conducted in Excel using the Student's test (unpaired, equal variance, two-tailed test) for comparisons with data that fit a normal distribution or the Mann-Whitney test for non-parametric comparisons.

ACKNOWLEDGEMENTS

We thank Gary Ward, Silvia Moreno, and Vern Carruthers for helpful discussions and suggestions, Florence Dzierszinski for the DsRed plasmid, Dominique Soldati for the Tet-repression system, and Keliang Tang for technical assistance. This work was supported by a predoctoral fellowship from the American Heart Association (SL) and a grant from the NIH (LDS).

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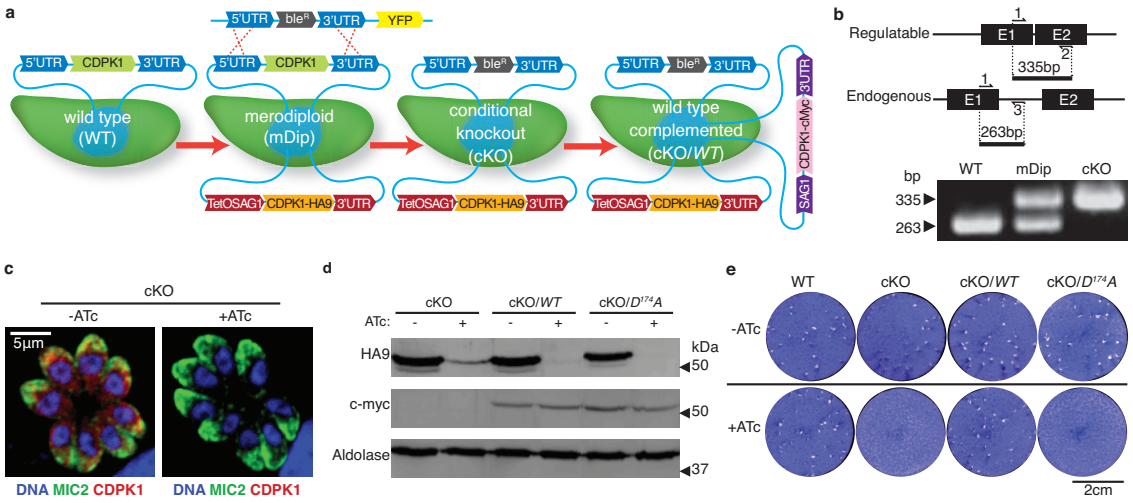


Figure 1. TgCDPK1 is essential for the parasite's lytic cycle. (**A**) Scheme for generating the TgCDPK1 conditional knockout (cKO) and the complemented strains. A regulatable, HA9-tagged copy of TgCDPK1 was first introduced into the wild-type strain (WT) to create a merodiploid (mDip). The endogenous TgCDPK1 was then replaced with a phleomycin resistance-cassette by homologous recombination to generate the cKO. Finally various constitutive, c-Myc tagged alleles were used to complement the cKO (denoted by cKO/"*allele*"). (**B**) Multiplexed polymerase chain reaction analysis of the endogenous and regulatable alleles of TgCDPK1. Primers 1, 2 and 3 were used together in all reactions. (**C**) Immunofluorescence analysis of the cKO grown for 72 h in the presence or absence of anhydrotetracycline (ATc), stained for the microneme protein MIC2 (green), HA9-tag (red) and DNA (blue). Micrographs were taken at identical exposure conditions by confocal microscopy. (**D**) Immunoblot of HA9-tagged regulatable and c-Myc-tagged constitutive TgCDPK1 in cKO and complemented strains grown 72 h in the presence or absence of ATc. Aldolase was included as a loading control. (**E**) Plaque formation in fibroblast monolayers in the presence or absence of ATc for 7 days.

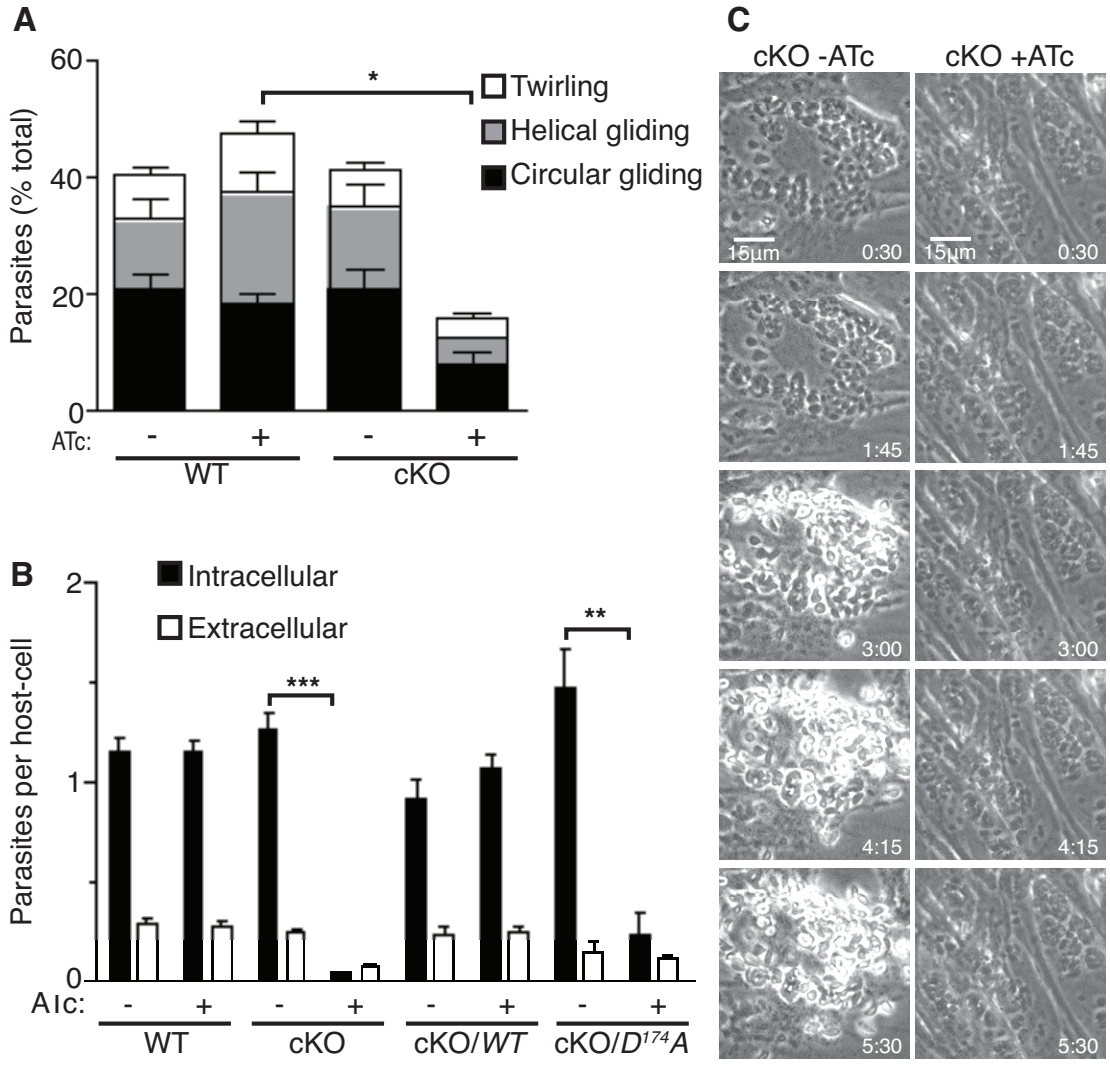


Figure 2. TgCDPK1 is required for phenotypes associated with microneme secretion. (A) Gliding motility quantified by video microscopy. Stacked bars represent the percentage of parasites moving, divided by the three types of movement. Student's *t* test; **P* < 0.05, mean ± s.e.m., *N* = 4 experiments. **(B)** Invasion of fibroblasts by WT, cKO and complemented strains. Extracellular and intracellular parasites were differentially stained and their numbers normalized to the number of host cells per field. Student's *t* test; ****P*<0.0005, ***P*<0.005, mean ± s.e.m., *N* = 3 experiments. **(C)** Ionophore-induced egress of cKO grown 72 hours in the presence or absence of ATc. Time stamp (min:sec) indicates time after calcium ionophore addition. See supplemental online videos.

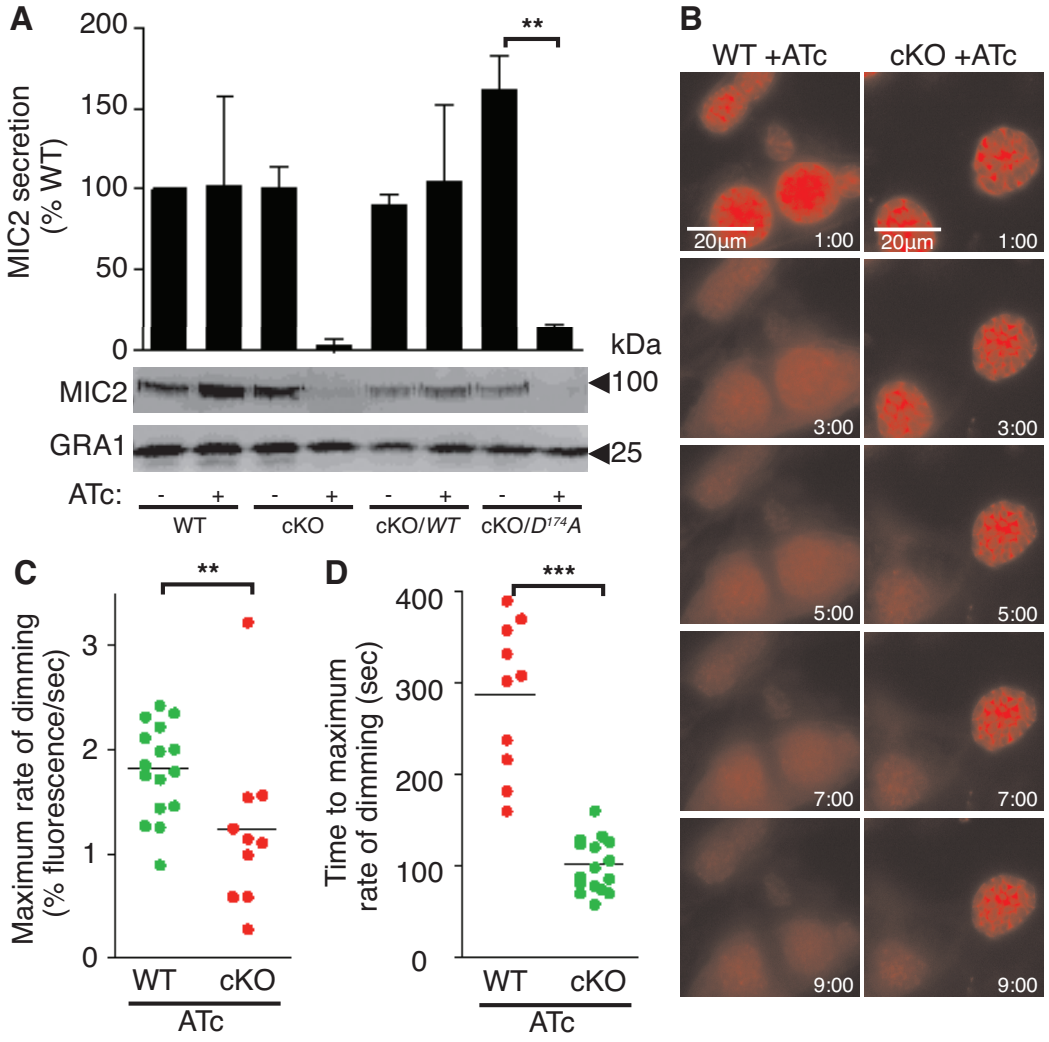


Figure 3. Calcium dependent secretion is blocked in the absence of TgCDPK1. (**A**) Secretion of the microneme protein MIC2 in response to a 15 min ethanol treatment, by strains grown in the presence or absence of ATc for 72 h. Secretion was detected by western blot, comparing secreted MIC2 in the supernatant to the total MIC2 from whole-cell lysates. Actin was used as a control for spontaneous lysis and GRA1 shows the constitutive secretion of dense granules by all strains. Bar graph represents secretion normalized to WT. Student's *t* test; $^{**}P < 0.005$, mean ± s.e.m., $N = 3$ experiments. (**B**) Ionophore-induced permeabilization of parasitophorous vacuoles of WT and cKO grown in the presence ATc for 90 h. Permeabilization was observed by fluorescence video-microscopy of parasites expressing a form of DsRed secreted into the vacuole. Time stamp (min:sec) indicates time after addition of calcium ionophore. All WT vacuoles were permeabilized in the period of observation, while 30% (4/14) of cKO vacuoles remained intact. (**C-D**) Quantification of the maximal rate of florescence loss and the time at which that rate occurred, for each vacuole observed to rupture. Mann-Whitney test; $^{***}P < 0.0005$, $^{**}P < 0.005$, mean, $N =$ 3 experiments.

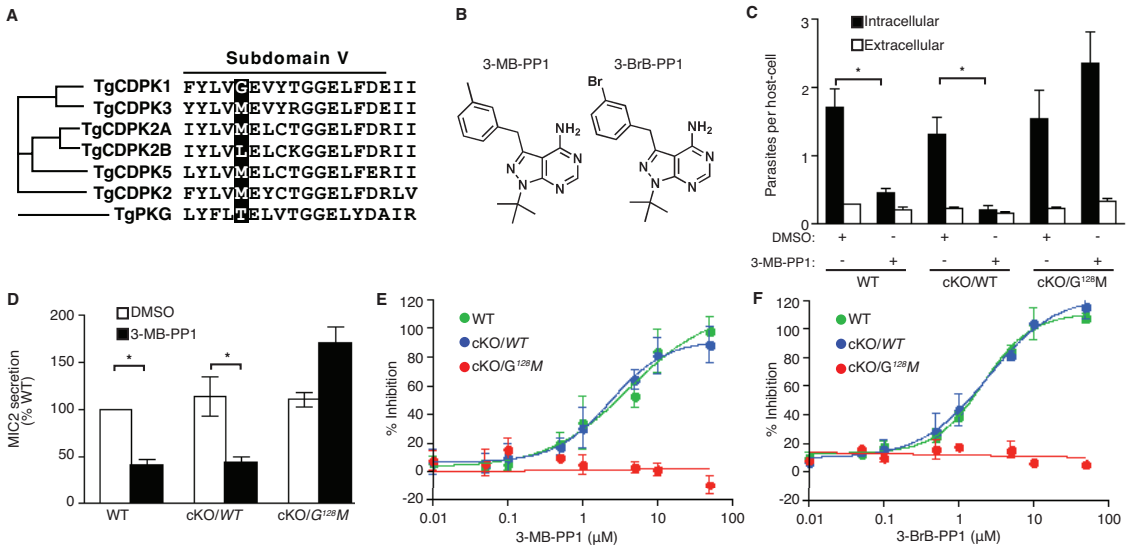
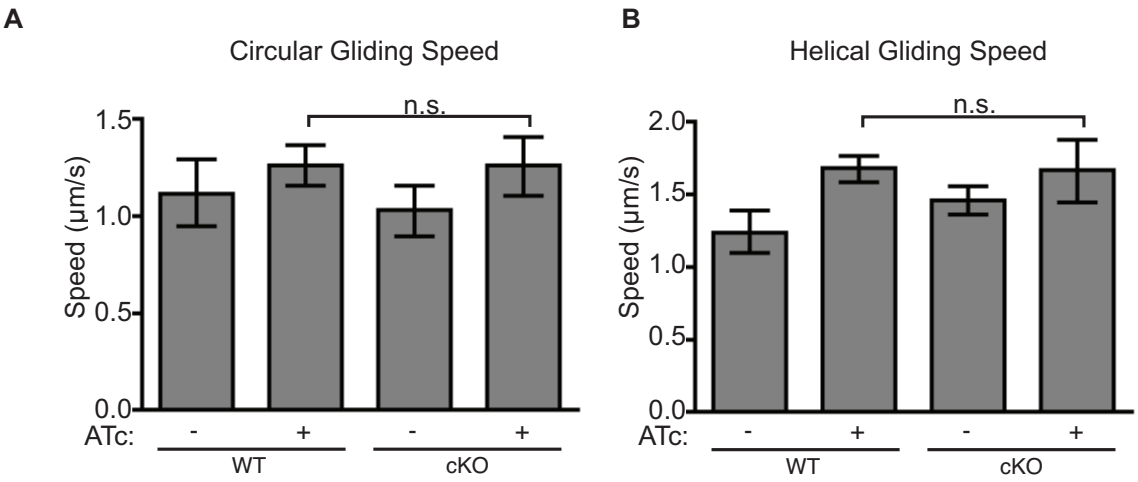
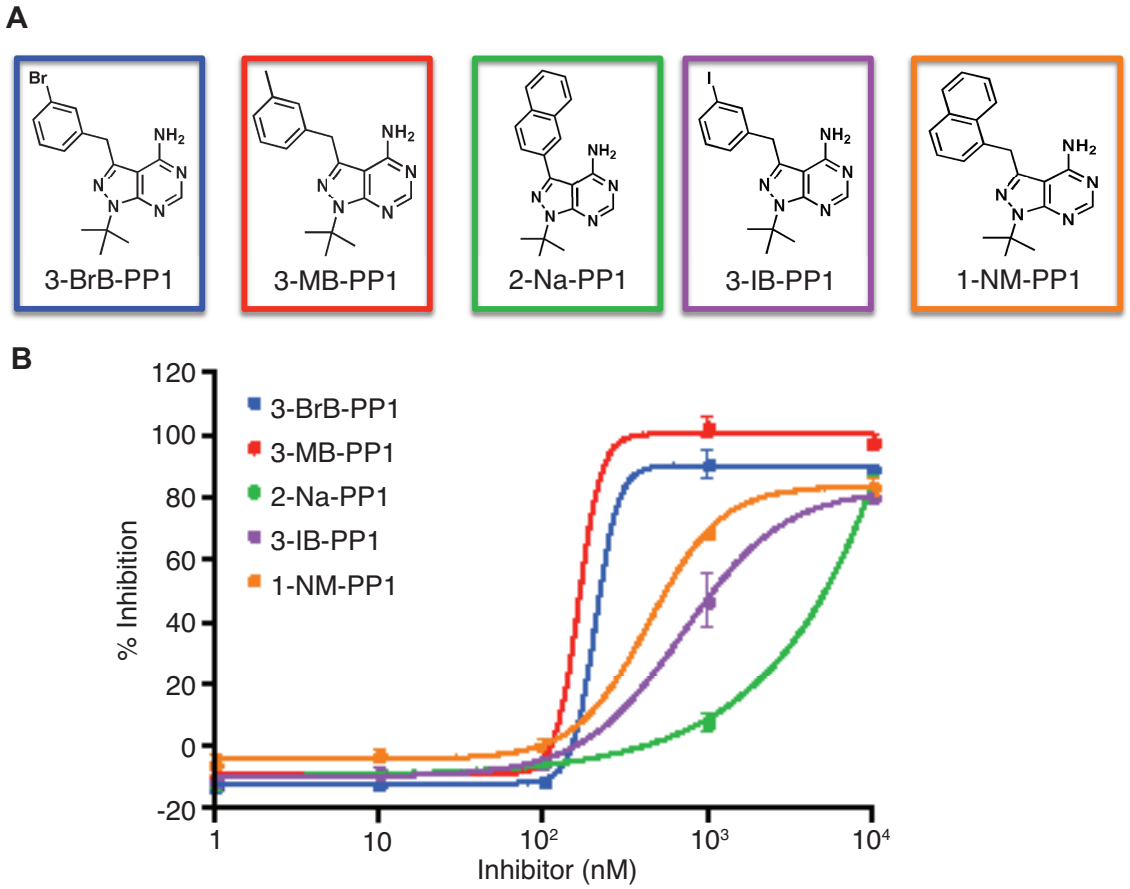


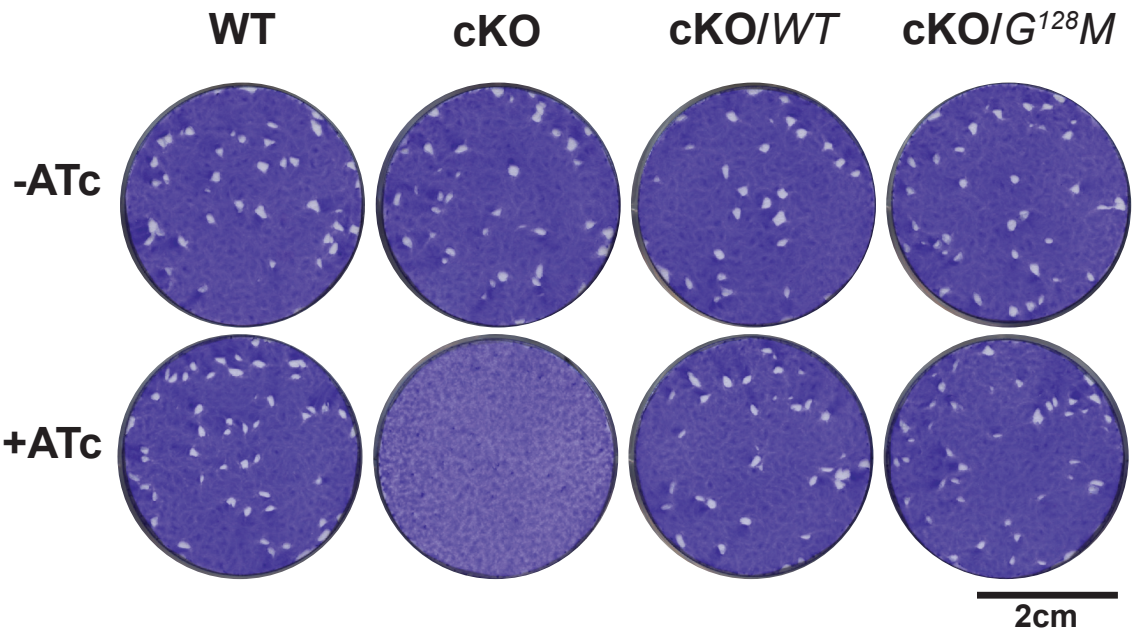
Figure 4. A C3-modified PP1 analogue specifically inhibits TgCDPK1 and blocks invasion and microneme secretion. (**A**) Alignment of the kinase sub-domain V, from the six canonical CDPKs of *T. gondii*. The gatekeeper residue is highlighted in the dark rectangle. CDPKs as defined previously **(Billker et al, 2009)**. (**B**) Structure of 3-MB-PP1. (**C**) 3-MB-PP1 significantly reduced invasion by WT and cKO/*WT*, but had no effect on cKO/$G^{128}M$. Drug diluent (DMSO) alone had no effect on invasion. Student's *t* test; $*P < 0.05$, mean ± s.e.m., $N = 3$ experiments. (**D**) Treatment with 3-MB-PP1 also reduced MIC2 secretion in strains carrying the *WT* allele of TgCDPK1, while cKO/$G^{128}M$ remained unaffected. Student's *t* test; $**P < 0.005$, mean ± s.d., $N = 3$ replicates.



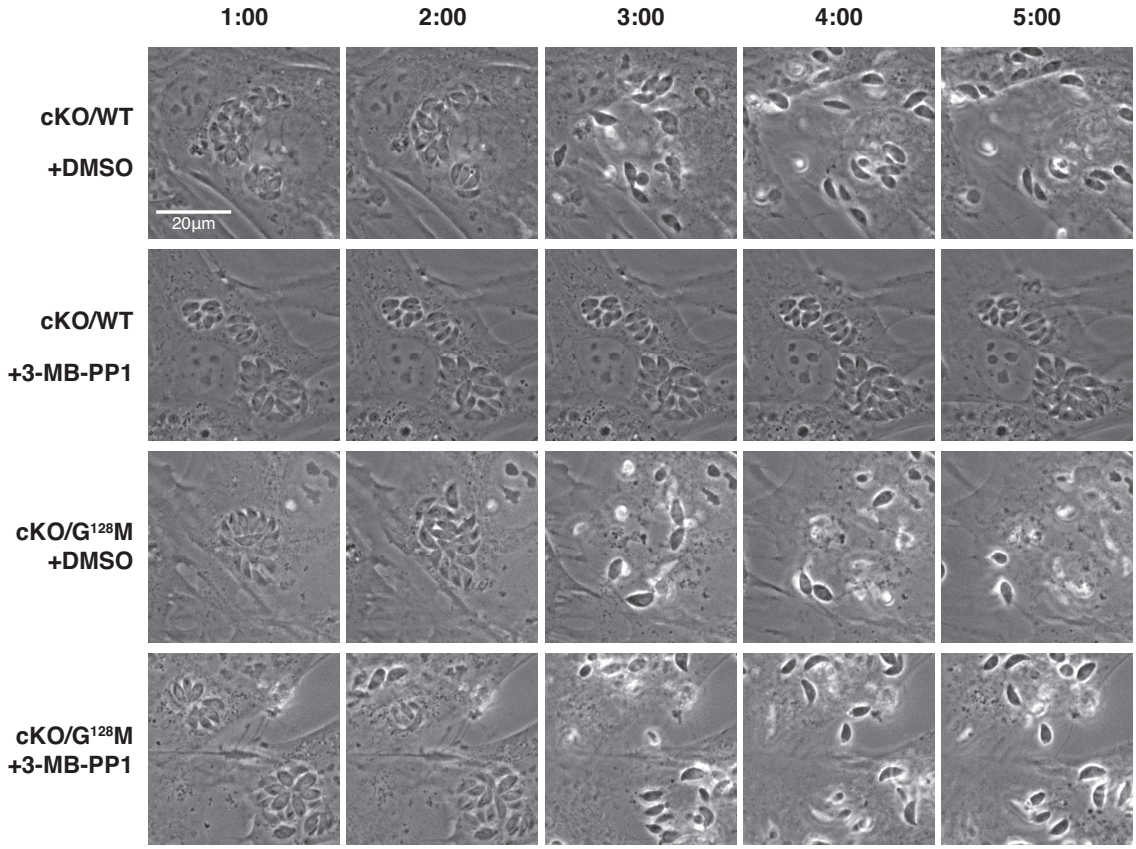
Supplementary Figure 1. cKO parasites capable of gliding after TgCDPK1 shutdown moved at the same speed as WT parasites under the same conditions. Gliding motility was quantified by video microscopy. Speed was calculated by measuring a parasite's displacement and dividing by the time taken for that displacement during (**A**), circular or (**B**), helical gliding. Student's *t* test; n.s. > 0.05, mean ± s.e.m., *N* = at least 5 independent trajectories.



Supplementary Figure 2. PP1-analogues inhibit host-cell lysis by *T. gondii*. (**A**) Chemical structure of the PP1 analogues tested. (**B**) Lysis of host cells after infection with WT parasites at an MOI of 1 in the presence of different drug concentrations. Values were normalized to uninfected wells (100%) and wells infected in the presence of vehicle (0%); mean ± s.d., representative experiment. 3-BrB-PP1: 1-tert-butyl-3-(3-bromo-benzyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine; 3-MB-PP1: 1-tert-butyl-3-(3-methyl-benzyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine; 2-Na-PP1: 1-tert-butyl-3-(2-naphthyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine; 3-IB-PP1: 1-tert-butyl-3-(3-iodo-benzyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine; 1-NM-PP1: 1-tert-butyl-3-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine.



Supplementary Figure 3. *WT* and *G*^{128}*M* alleles under the regulation of the TgCDPK1 endogenous promoter can complement the TgCDPK1 cKO. Plaque formation in fibroblast monolayers in the presence or absence of ATc for 8 days.



Supplementary Figure 4. 3-MB-PP1 can block egress in parasites expressing *WT* but not *G^{128}M* alleles of TgCDPK1. Ionophore-induced egress of strains pre-treated with either 3-MB-PP1 or vehicle (DMSO) for 25 min. Time stamp (min:sec) indicates time after calcium ionophore addition.

Strain	Experiment	Total Vacuoles	Egressed Vacuoles	AverageTime to Egress (min)
cKO - ATc	1	12	12	2.3 ± 0.2
	2	17	17	2.6 ± 0.2
	3	13	13	2.8 ± 0.6
cKO + ATc	1	10	0	> 9.5
	2	17	0	>9.5
	3	18	1	>9.5

Supplementary Table 1. Egress of parasites from host cells following ionophore stimulation. Ionophore-induced egress of cKO parasites grown in the presence or absence of ATc for 72 h. Mean average time to egres ± s.d.

Enzyme	Gatekeeper Residue	IC_{50} (nM)
TgCDPK1	$G^{128}G$	3
	$G^{128}M$	$>10^4$
c-Src	$T^{338}T$	2000
	$T^{338}G$	3
	$T^{338}A$	10

Supplementary Table 2. *In vitro* IC_{50} of 3-MB-PP1 against recombinant enzymes. In vitro sensitivity of different kianses to 3-MB-PP1 shows that small gatekeeper residues (glycine or alanine) render kinases significantly more sensitive than those harboring bulkier residues (methionine or threonine). IC_{50} values were determined for *WT* and mutant recombinant proteins from a dose response curve as described in the materials and methods.

CHAPTER III

Distinct Signaling Pathways Control *Toxoplasma* Egress and Host-Cell Invasion

PREFACE

Work presented in this chapter was conducted by Sebastian Lourido and Keliang Tang. SL designed and performed the majority of experiments, analyzed the data, and generated the figures. KT produced recombinant proteins and performed in vitro kinase assays. The first draft of this chapter was written by SL. Comments from L. David Sibley were incorporated into the final version presented here. This manuscript has been submitted for publication in its current form.

ABSTRACT

Calcium signaling coordinates motility, cell invasion, and egress by apicomplexan parasites, yet the key mediators that transduce these signals remain largely unknown. One underlying assumption is that invasion into and egress from the host cell depend on highly similar systems to initiate motility. Using a chemical-genetic approach to specifically inhibit select calcium-dependent kinases (CDPKs), we instead demonstrate that these pathways are controlled by different kinases: both TgCDPK1 and TgCDPK3 were required during ionophore-induced egress, but only TgCDPK1 was required during invasion. Similarly, microneme secretion, which is necessary for motility during both invasion and egress, universally depended on TgCDPK1, but only exhibited TgCDPK3 dependence when triggered by certain stimuli. We also demonstrate that egress comes under a further level of control by cyclic GMP-dependent protein kinase and that its activation can directly induce egress and overcome the inhibition of TgCDPK3. These results demonstrate that separate signaling pathways are integrated to regulate motility in response to the different signals that promote invasion or egress during infection by *T. gondii*.

INTRODUCTION

Toxoplasma gondii belongs to the phylum Apicomplexa, a group of single-celled, obligate intracellular parasites of animals, including *Plasmodium spp.*, the etiological agents of malaria. Apicomplexans are characterized by a specialized complex of apical organelles used to penetrate and modify the host cells they replicate within (Besteiro et al, 2011). Host cell invasion is powered by parasite motility and relies on the posterior translocation of adhesins from their site of secretion at the apical end of the parasite (Sibley, 2010). The signaling pathways that regulate parasite motility are poorly understood, although transient increases in cytosolic calcium are known to be essential for microneme secretion, motility, and host cell invasion (Carruthers & Sibley, 1999; Lovett & Sibley, 2003). In *T. gondii*, it was first demonstrated using the calcium ionophore A23187, that direct manipulation of the intracellular calcium concentrations could trigger motility and induce egress (Endo et al, 1982). However, these calcium transients can be triggered naturally by the accumulation of abscisic acid produced by parasites (Nagamune et al, 2008), or exposure to a low potassium environment, which occurs upon host cell rupture (Moudy et al, 2001). Artificially, intracellular calcium levels can be increased and microneme secretion induced by incubating *T. gondii* parasites with ethanol (Carruthers et al, 1999), which occurs through IP_3 generation (Lovett et al, 2002). In *Eimeria tenella*, a related apicomplexan, microneme secretion can be triggered by serum albumin (Bumstead & Tomley, 2000; Wiersma et al, 2004), which also stimulates motility in *Plasmodium berghei* sporozoites, in a manner dependent on intracellular calcium (Kebaier & Vanderberg, 2010). At a different point in the *Plasmodium* life cycle, xanthurenic acid in the mosquito midgut can also trigger calcium transcients in male gametocytes and induce their differentiation (McRobert et al, 2008). Taken together, these observations suggest that calcium acts as a second messenger in response to a variety of extracellular signals, in many cases converging on the regulation of motility.

The wide range of cellular processes regulated in most eukaryotes by intracellular calcium occurs in part through the activation of calcium-regulated protein kinases. In animals, these kinases belong to two families: protein kinase C (PKC) and related kinases, and calmodulin-dependent kinases (CaMKs) (Barclay et al, 2005). However, plants and alveolates lack obvious PKC homologues, and calcium-dependent protein kinases (CDPKs) represent the dominant calcium-responsive kinases in these organisms (Harper & Harmon, 2005; Nagamune & Sibley, 2006). CDPKs are characterized by a kinase domain followed by a calmodulin-like domain that directly binds calcium to activate the enzyme. CDPKs have a novel mechanism of activation that is triggered by binding of calcium to the EF hands, causing a reorganization of the calcium-binding domain that releases the kinase domain from inhibition (Wernimont et al, 2010). In *Arabidopsis*, as many as 42 CDPK isoforms regulate myriad transcriptional and cellular processes, leading to effects in diverse phenotypes including pollen tube formation, closure of stomata, and a wide range of stress responses (Harper & Harmon, 2005).

Apicomplexan parasites contain an expanded family of CDPKs and several of these have been shown to control important functions (Billker et al, 2009). *Plasmodium spp.* and *T. gondii* genomes each code for 5 and 6 canonical CDPKs respectively, and an additional 2 to 6 related kinases with different domain architectures (Billker et al, 2009). In *Plasmodium berghei*, disruption of CDPKs that are not essential in the asexual phase leads to developmental blocks at later stages, including male gamete exflagellation (*PbCDPK4*), ookinete motility (*PbCDPK3*), and sporozoite invasion of hepatocytes (*PbCDPK6*) (Billker et al, 2004; Coppi et al, 2007; Ishino et al, 2006). However, this approach is limited to kinases dispensable for the asexual cycle, and the block caused by disruption of a particular kinase precludes examination of its function beyond the stage of developmental arrest. This limitation was recently circumvented by tagging the endogenous *Plasmodium falciparum* CDPK5 with a destabilization domain, which allowed investigators to regulate

its degradation, demonstrating a role for PfCDPK5 in merozoite egress from erythrocytes (Dvorin et al, 2010). In *T. gondii*, the availability of a regulatable promoter (Meissner et al, 2002) allowed us to generate a conditional knockout of *TgCDPK1*, thus revealing its requirement for microneme secretion, and hence motility and invasion (Lourido et al, 2010). We also exploited the unique ATP-binding pocket of TgCDPK1, which has a glycine at a key position termed the 'gatekeeper', to specifically inhibit TgCDPK1 using bulky pyrazolo [3,4-*d*] pyrimidine (PP) derivatives (Lourido et al, 2010), confirming the role of TgCDPK1 using chemical genetics.

The function of CDPKs has also been probed *in vitro* using kinase assays that suggest that components of the motor complex, which is essential for motility (Meissner et al, 2001), are phosphorylated by PfCDPK1 in *P. falciparum* (Green et al, 2008; Ridzuan et al, 2012). However, PfCDPK1 remains refractory to genetic disruption in *P. falciparum*, and there is no test of whether the motor complex is an essential target of PfCDPK1 *in vivo*. Here, we have extended the chemical-genetic approach to probe the function of TgCDPK3, which is the closest homologue of PfCDPK1 (Billker et al, 2009). Our results suggest a much broader role for this kinase in responding to signals that govern motility, and yet which likely do not involve direct regulation of the motor complex.

RESULTS

TgCDPK3 Localizes to the Parasite Membrane

PfCDPK1 has been reported to localize to the membrane in *P. falciparum* merozoites (Green et al, 2008). To localize CDPK3 in *T. gondii*, we introduced a C-terminally HA9-tagged allele of *TgCDPK3*, under the regulation of a modified *TetO7SAG1* promoter, into the TATi strain (Meissner et al, 2002). Immunofluorescence analysis of the localization of TgCDPK3 showed that it localizes to the membrane of the parasite in a pattern similar to the surface antigen SAG1 (Figure 1). The N-terminal residues of TgCDPK3 and its homologue PfCDPK1 are predicted to be acylated and therefore likely required for its localization. We mutated the predicted myristoylation site (G^2) and palmitoylation site (C^3) to alanine, alone or in combination. We observed that mutation of either putative acylation site was sufficient to mislocalize TgCDPK3 to the parasite cytosol (Figure 1). In all the mutants there was also reduced deposition of TgCDPK3 in the trails of gliding parasites, also consistent with a lack of membrane localization (Figure 1). Unfortunately, we were unable to disrupt the endogenous copy of *TgCDPK3*, despite the presence of a second wild type regulatable copy, pointing to the potential limitations of this strategy.

Chemical Genetic Manipulation of CDPKs

The ATP-binding pockets of many eukaryotic protein kinases can be engineered to be susceptible to PP derivatives, which are normally excluded due to the presence of a bulky residue at a key position in the binding pocket, the aforementioned gatekeeper (Bishop et al, 2000). An alignment of the kinase domains from the 109 *T. gondii* kinases that are predicted to be active (Peixoto et al, 2010) shows that more than 80% contain a large hydrophobic amino acid as the gatekeeper residue (green bars; Figure 2A). Unique among the active kinases, TgCDPK1 harbors a glycine at this key position. Previous studies demonstrated that susceptibility of TgCDPK1 to 3-methyl-benzyl pyrazolo [3,4-*d*]

pyrimidine (3-MB-PP1) and related compounds, both *in vivo* and *in vitro*, depends on the gatekeeper residue (Lourido et al, 2010; Murphy et al, 2010; Sugi et al, 2010). To determine whether the related kinase TgCDPK3 could be rendered susceptible to selective inhibition by PP derivatives, we generated recombinant proteins for full-length TgCDPK3 with either a glycine or a methionine at the gatekeeper positions (designated by a superscript G or M, respectively) and compared them to similar allelic forms of TgCDPK1. All four enzymes were similarly able to phosphorylate the synthetic substrate peptide syntide-2, but showed dramatically different susceptibilities to 3-MB-PP1. As previously shown, wild-type TgCDPK1 (CDPK1^{G}) showed sub-nanomolar susceptibility to the inhibitor (IC_{50} = 0.8 nM, 95% CI [0.3 to 2.3]), and mutation of the gatekeeper to a methionine (CDPK1^{M}) rendered the enzyme insensitive to inhibitor concentrations of up to 1mM (green lines; Figure 2B). Conversely, wild-type TgCDPK3 (CDPK3^{M}) was insensitive to the inhibitor unless its gatekeeper residue was mutated to a glycine (CDPK3^{G}), in which case the enzyme became sensitive, similarly to wild-type TgCDPK1 (IC_{50} = 19.2 nM, 95% CI [8.5 to 43.5]; red lines; Figure 2B).

In order to study the function of TgCDPK1 and TgCDPK3 *in vivo*, we introduced similar gatekeeper residues into the genes encoding these enzymes in parasites. These manipulations were made easier by the high levels of homologous recombination in strains lacking *Ku80*, an essential component of the non-homologous end-joining DNA repair pathway (Fox et al, 2009; Huynh & Carruthers, 2009). Using the *Ku80* knockout as the parental strain, henceforth designated as CDPK1^{G}, we replaced the wild-type *TgCDPK1* with a partial cDNA coding for a methionine at the gatekeeper position and c-terminally tagged with c-myc (CDPK1^{M}; Figure 2C). The CDPK1^{M} strain was further used to manipulate the *TgCDPK3* locus. The later manipulations were performed using a vector that lacked a promoter and contained the second intron of *TgCDPK3* fused to the cDNA starting at the third exon and ending with a c-terminal Ty-tag (Figure 2C). By selecting for

drug resistance conferred by the vector, and screening for homologous recombination at the second intron by PCR, we were able to isolate parasites that expressed a single functional Ty-tagged copy of *TgCDPK3*, downstream of which the endogenous allele was left without a promoter or the first two exons, and was therefore silent. In this manner, we isolated strains where the Ty-tagged CDPK3 resembled the wild type and carried a methionine at the gatekeeper position ($CDPK3^{M}$; Figure 2C), or where the position had been mutated to a glycine ($CDPK3^{G}$; Figure 2C). Both alleles of CDPK3 were expressed to similar levels, as demonstrated by western blotting against the Ty-tag (Figure 2D), in the otherwise isogenic background of the $CDPK1^{M}$ strain.

TgCDPK3 is Dispensable for Invasion but Required for Egress

The isogenic parasite lines expressing either sensitive or resistant alleles were used to investigate whether inhibition of CDPK3 affected host cell invasion, a process previously shown to depend on the related enzyme TgCDPK1 (Lourido et al, 2010). Consistent with our previous results, the strain carrying the wild-type *TgCDPK1* allele was significantly impaired in its ability to invade in the presence of 3-MB-PP1 ($CDPK1^{G}$; Figure 3A). Allelic replacement of the endogenous allele for one carrying a methionine at the gatekeeper position rendered the $CDPK1^{M}$ strain completely resistant to inhibition (Figure 3A). In contrast to the observations with TgCDPK1, 3-MB-PP1 had no effect on the $CDPK3^{G}$ strain carrying the sensitive allele of the kinase (Figure 3A).

To determine whether TgCDPK3 was also dispensable for other phenotypes previously associated with TgCDPK1 inhibition, we examined the stimulation of egress from infected host cells by the calcium ionophore A23187. As previously shown, treatment with 3-MB-PP1 significantly decreased the ability of the $CDPK1^{G}$ strain to egress from host cells in response to A23187 (Figure 3B). In contrast to the lack of a role during invasion, inhibition of TgCDPK3 within intracellular parasites dramatically inhibited their ability to respond to A23187, with an IC_{50} for 3-MB-PP1 of 0.2mM (95% CI [0.1, 0.5]), ten-fold

lower than that which showed no effect on invasion by the same strain ($CDPK3^{G}$; Figure 3B).

To further investigate the role of TgCDPK3 during egress, we monitored the permeabilization of the parasitophorous vacuole (PV) membrane following stimulation of intracellular parasites with A23187. This was achieved by transiently expressing a constitutively secreted form of DsRed, which accumulates in the PV and is released prior to egress, following membrane permeabilization that occurs due to microneme secretion and release of a perforin-like pore forming protein called TgPLP1 (Kafsack et al, 2009). Ionophore stimulation of the $CDPK3^{M}$ strain treated with 3-MB-PP1 resulted in normal permeabilization of the PV membrane and egress within a few minutes, consistent with this strain being resistant to PP inhibitors (Figure 3C). In contrast, the $CDPK3^{G}$ strain treated with 3-MB-PP1 failed to permeabilize the PV membrane and remained immobile for the entire recorded period, up to 10 min following the addition of ionophore (Figure 3C). To further quantify the differences in timing and magnitude of this response, we measured the relative fluorescence of a circular area within the PV. DsRed fluorescence was completely lost from $CDPK3^{M}$ vacuoles within 3 min, but remained stable in $CDPK3^{G}$ strains for the entire period recorded (Figure 3D). Together these findings highlight the striking requirement for both TgCDPK1 and TgCDPK3 for egress, while only TgCDPK1 is essential during invasion.

Inhibition of TgCDPK1 or TgCDPK3 Leads to Defects in Gliding Motility and Microneme Secretion

Having uncovered a distinction between the effects of TgCDPK1 *vs*. TgCDPK3 on egress and invasion, we asked whether the processes of motility and microneme secretion underlying these phenotypes were also differentially regulated. Analysis of gliding motility in all four strains in the presence of 3-MB-PP1 demonstrated a requirement for both

TgCDPK1 and TgCDPK3 in this process. Inhibition of either kinase led to an approximate 50% reduction in the number of parasites moving during the period observed (Figure 4A). Similar the previous report that TgCDPK1 is required for motility (Lourido et al, 2010), all types of motility were less frequent when TgCDPK3 was inhibited. However once movement was initiated, the pattern and speed of parasite movement appeared normal.

Parasite motility also requires release of adhesins from micronemes (Carruthers & Sibley, 1999), a process governed by TgCDPK1 (Lourido et al, 2010). Hence, we examined the role of TgCDPK3 in release of the micronemal protein MIC2 following stimulation with ethanol to increase intracellular calcium concentrations. We compared the supernatants of parasites treated with 3-MB-PP1 or a vehicle control (DMSO) and found that, although the $CDPK1^{G}$ strain was significantly inhibited in its response to ethanol, the $CDPK3^{G}$ strain secreted MIC2 to the same extent as the inhibitor-resistant $CDPK3^{M}$ strain (black bars; Figure 4B).

Addition of serum can also trigger secretion, either alone or in combination with ethanol (Bumstead & Tomley, 2000; Kebaier & Vanderberg, 2010), and we tested the requirements for TgCDPK1 or TgCDPK3 in responding to this combination of signals. Once again, inhibition of TgCDPK1 significantly reduced secretion of MIC2 into the supernatant ($CDPK1^{G}$; Figure 4B). However, in contrast to the results from ethanol alone, in the presence of FBS inhibition of TgCDPK3 by 3-MB-PP1 treatment significantly reduced secretion of MIC2 ($CDPK3^{M}$; Figure 4B). To confirm that the secretion defect observed in response to TgCDPK3 inhibition was a result of microneme secretion and not due to inhibition of proteolytic activity leading to reduced shedding of protein into the supernatant, we measured the amount of MIC2 on the parasite surface by FACS. Surface accumulation was not observed for any of the strains, regardless of whether TgCDPK3 was inhibited or not (Figure 4C). In contrast, shutdown of the ROM4 protease, which cleaves MIC2 from the surface (Buguliskis et al, 2010), led to an accumulation of MIC2 on the

parasite surface (ROM4 cKO + ATc; Figure 4C). These findings confirm that the observed defect in MIC2 secretion caused by TgCDPK3 inhibition is a consequence of reduced microneme secretion, and not loss of protein shedding from the parasite surface.

Activation of PKG Can Overcome the Inhibition of TgCDPK3 During Egress

Given our observations that microneme secretion required TgCDPK3 under certain conditions but not others, we hypothesized that another kinase might compensate for TgCDPK3. In *T. gondii* and other apicomplexans, use of a potent cyclic GMP-dependent protein kinase (PKG) inhibitor, known as Compound 1, has been shown to affect microneme secretion, motility, and invasion of tachyzoites (Donald et al, 2006; Gurnett et al, 2002; Wiersma et al, 2004). Given that a bulky gatekeeper residue also confers resistance to Compound 1, we used the CDPK1^{M} strain to assess the specific inhibition of PKG, without concern for potential inhibition of wild-type TgCDPK1. We first sought to establish whether PKG was playing a role in A23187-induced egress, by comparing the ability of strains to egress following incubation with different concentrations of Compound 1. Consistent with a role for PKG in egress that is independent of TgCDPK1, both CDPK1^{G} and CDPK1^{M} strains showed marked susceptibility to inhibition by Compound 1 (Figure 5A).

Having established a role for PKG in egress, we wondered whether stimulation of the PKG pathway could on its own induce egress. In *P. berghei*, male gametocyte exflagellation can be artificially triggered by high concentrations (0.4 mM) of a phosphodiesterase inhibitor called Zaprinast, which causes cyclic GMP to accumulate, leading to PKG activation (Billker et al, 2004; McRobert et al, 2008). We tested the ability of PKG activation to induce egress by incubating intracellular parasites with 0.5 mM Zaprinast and monitoring their behavior by videomicroscopy. We observed that within minutes of adding Zaprinast, parasites became motile and egressed from host cells, with similar kinetics to the A23187-induced phenomenon (Figure 5B). To confirm that Zaprinast-induced egress

works through the activation of PKG, we tested whether Compound 1 could inhibit this process. Compound 1 completely blocked the ability of Zaprinast to trigger egress, with an IC_{50} of 0.6 mM (95% CI [0.5, 0.7]; Figure 5C), demonstrating that activation of PKG is both necessary and sufficient for egress.

Studies in *P. berghei* ookinetes have shown that stimulation of the PKG pathway can partially compensate for loss of ookinete motility controlled by PbCDPK3, which is not an orthologue of TgCDPK3 but instead is unique to *Plasmodium*, despite bearing a similar name for historical reasons (Moon et al, 2009). To determine whether stimulation of PKG could overcome the inhibition of TgCDPK3, we added Zaprinast to intracellular parasites in the presence of 3-MB-PP1 and examined egress after 20 min. We observed that high concentrations of Zaprinast were able to overcome the inhibition of TgCDPK3 ($CDPK3^{G}$; Figure 5D), indicating that stimulation of PKG can compensate for the role of CDPK3 in egress of *T. gondii*. Comparing the levels of egress triggered by either Zaprinast or A23187 showed that inhibition of $CDPK3^{G}$ by 3-MB-PP1 was almost completely rescued by activation of the PKG pathway with Zaprinast (Figure 5E). Furthermore, Zaprinest was more effective in triggering egress of parasites expressing the 3-MP-PP1 insensitive $CDPK3^{M}$ allele, (EC_{50} = 93 mM, 95% CI 82 - 106) *vs.* the sensitive $CDPK3^{G}$ allele (EC_{50} 246 mM, 95% CI 173 - 349), suggesting that these pathways work together.

DISCUSSION

We tested the previously proposed model that TgCDPK3 regulates the motor complex required for motility using a highly specific chemical-genetic approach. Our findings are consistent with a role for TgCPDK3 in activating parasite motility, rather than acting on the motor complex directly. Specific chemical inhibition of sensitized alleles revealed that TgCDPK1 was required for both egress and invasion, while TgCDPK3 was required only for egress. Similarly, microneme secretion, which is necessary for both egress and invasion, required TgCDPK1 under all circumstances but only exhibited TgCDPK3 dependence under specific circumstances. We also revealed an unexpected role for PKG in egress from host cells, and demonstrated that stimulation of PKG can overcome the inhibition of TgCDPK3. These results illustrate that egress is under very tight control of three distinct kinases, each of which is essential for this process. The fact that under certain conditions the functions of TgCDPK3 can be circumvented by the activation of PKG, suggests that the parasite modulates input from different signals to fine-tune responses using a complex network of signaling proteins.

We previously reported that TgCDPK1 is specifically inhibited by PP analogues due to the natural occurrence of a glycine at its gatekeeper position, a feature unique among all the active kinases in *T. gondii* (Lourido et al, 2010). In budding yeast, the bio-orthogonality of these PP analogues has allowed researchers to study the function of various kinases by mutating their gatekeeper residues to render them sensitive to inhibition (Bishop et al, 2000; Snead et al, 2007). Seeking to extend this chemical-genetic approach to parasite kinases, we replaced the endogenous TgCDPK1 with an allele harboring a methionine at the gatekeeper position to generate a resistant genetic background in which to sensitize other kinases. Using this background, we replaced the *TgCDPK3*^{M} allele with TgCDPK^{G}, which is sensitive to 3-MB-PP1, in order to study its function. This manipulation of the *TgCDPK3* allele did not affect parasite replication or viability, demonstrating that this

is a valid strategy for studying essential parasite kinases. By preserving the endogenous promoter, this strategy does not significantly alter the timing or level of expression of the kinase, which is in contrast unavoidable with the regulatable promoters currently available for *T. gondii*. In the case of TgCDPK3, it is possible that such differences in protein expression between the endogenous and regulatable alleles might have led to fitness costs that precluded the isolation of conditional knockouts from mixed populations. Another advantage of the chemical genetic approach over conventional conditional alleles is that it allows for rapid inhibition of these essential kinases, excluding the possibility of pleiotropic effects from loss of the kinase during development. Our results suggest that this strategy could be generalizable to other parasite kinases given the conservation of the ATP-binding pocket in most eukaryotic serine/threonine protein kinases.

Using this chemical-genetic strategy, we were able to compare the functions of TgCDPK1 and TgCDPK3 at different stages of the *T. gondii* life cycle. Inhibition of either TgCDPK1 or TgCDPK3 completely prevented egress in response to the calcium ionophore A23187, while only TgCDPK1 was required for invasion. Together these data provide the first evidence in *T. gondii* that the signaling requirements for invasion differ from those for egress. In contrast, most cellular processes governing motility are thought to do so globally, such as the actomyosin motor complex (Soldati & Meissner, 2004) or the secretion of adhesins from micronemes (Lourido et al, 2010). A similar observation of the requirement of *P. falciparum* CDPK5 in egress but not invasion of red blood cells (Dvorin et al, 2010) indicates an emerging theme distinguishing the signaling events required for these two processes in apicomplexans. Whether the signaling differences during egress and invasion reflect the regulation of known processes by different pathways, or the regulation of still unknown cellular responses required for egress but not invasion, will be the focus of future research. Such studies may be facilitated by the use of sensitized kinases, which we have

demonstrated successfully for CDPKs, and techniques for downstream analysis of their specific targets, as described previously (Allen et al, 2005; Snead et al, 2007).

Inhibition of TgCDPK1 or TgCDPK3 reduced gliding motility of extracellular parasites to the same extent. We had previously proposed that because TgCDPK1 controls microneme secretion, it is universally required for motility during egress and invasion. In contrast, the phenotypes described for TgCDPK3 are an exception to this all-or-none model of microneme secretion. Intracellular parasites demonstrated a strong requirement for TgCDPK3 in microneme secretion as shown by the lack of PV membrane permeabilization in response to A23187. Release of DsRed from the PV normally occurs when TgPLP1 is secreted from micronemes, an early event that is necessary for ionophore induced egress (Kafsack et al, 2009). Our findings indicate that TgCDPK1 and TgCDPK3 are both required for microneme release when parasites are intracellular. However, the ability of parasites to invade host cells normally following TgCDPK3 inhibition argued against a defect in microneme secretion during this process. Direct measurement of MIC2, one of the adhesins released from micronemes, demonstrated that the two kinases were also differentially required for microneme secretion in response to different stimuli, similarly displaying a universal requirement for TgCDPK1 but a stimulus-specific requirement for TgCDPK3. Although the signaling pathways regulated by ethanol and FBS are still poorly understood, as is their relationship to the natural stimulation of microneme secretion, our ability to distinguish between these two kinases suggests that TgCDPK3 signaling is only required for microneme secretion under certain conditions.

In *P. falciparum*, the ortholog of TgCDPK3, PfCDPK1, has been implicated in the phosphorylation of GAP45 and MTIP (Green et al, 2008), components of the actomyosin motor complex that are conserved in apicomplexans (Frenal et al, 2010) and required for motility and host cell invasion. However, these studies only establish that sites phosphorylated by PfCDPK1, under permissive conditions *in vitro,* can also be identified

in parasites, while failing to exclude the potential role of other kinases in this process. Additionally, recent studies have failed to demonstrate a functional role for some of the phosphorylation sites on GAP45, showing that they are not required for the localization or assembly of the motor complex, functions previously attributed to these modifications (Ridzuan et al, 2012). From our own results, three observations argue against a role for TgCDPK3 in the regulation of the motor complex: (*i.*) microneme secretion is independent of the motor complex (Kafsack et al, 2009; Meissner et al, 2002) yet under some conditions it was dependent on TgCDPK3, (*ii.*) although the fraction of parasites moving was reduced upon TgCDPK3 inhibition, gliding appeared normal once initiated, and (*iii.*) inhibition of TgCDPK3 did not affect invasion, which requires a functioning motor complex (Dobrowolski & Sibley, 1996; Meissner et al, 2002). Although we cannot rule out that under certain conditions components of the motor complex are phosphorylated by TgCDPK3, such an activity would have to be either dispensable or performed by a different kinase during invasion. Instead, our results indicate that TgCDPK3 regulates other cellular targets to activate motility.

The involvement of PKG in ionophore-induced egress, extends previous observations linking this kinase to microneme secretion and host-cell invasion in apicomplexans (Wiersma et al, 2004). Notably, this is the first report that PKG stimulation using Zaprinast can, on its own, induce egress of *T. gondii* from host cells. Activation of PKG was also able to overcome the inhibition of TgCDPK3, although the shift in EC_{50} for Zaprinast when TgCDPK3 was active *vs*. inactive suggests that the two pathways likely operate cooperatively. In *Plasmodium*, Zaprinast has been shown to stimulate male gametocyte exflagellation, which is also regulated by CDPKs (McRobert et al, 2008). In *P. berghei*, genetic deletion of the phosphodiesterase that degrades cyclic GMP, thus stimulating the PKG pathway, has been shown to overcome the block in ookinete motility caused by loss of PbCDPK3, a CDPK unique to *Plasmodium* (Moon et al, 2009). PKG and CDPKs

belong to related groups of kinases, whose targets consist of sites enriched in basic amino acids preceding S/T, opening the possibility that they might display overlapping substrate specificities (Hanks & Hunter, 1995). The ability of different kinases to converge on a single target allows different stimuli to regulate a single pathway, and is a known feature of the nucleotide-activated family of kinases including PKG (Pearce et al, 2010). Taken together, these observations suggest that PKG acts in parallel to various CDPK-regulated pathways, providing multiple layers of regulation on a single cellular process, and allowing the parasite to respond differently to intracellular signals that govern egress *vs*. extracellular signals that control invasion.

MATERIALS AND METHODS

Parasite Growth and Selection

T. gondii tachyzoites were grown in human foreskin fibroblasts (HFF) cultured in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen) supplemented with 10% tetracycline-free FBS (HyClone), 2 mM glutamine, 10 mM HEPES (pH 7.5), and 20 g/ml gentamicin ,as previously described (Lourido et al, 2010). When noted, parasites were selected using growth media containing any of the following: chloramphenicol (20 g/ml; Sigma), phleomycin (5 g/ml; Invitrogen), ATc (1 g/ml; Clontech), pyrimethamine (3 mM; Sigma), 25 g/ml mycophenolic acid, 50 g/ml xanthine. Negative selection of *HXGPRT* was performed by growing parasites in DMEM supplemented with 1% dialyzed FBS (Invitrogen), 10mM HEPES (pH 7.4) and 340 g/ml 6-thioxanthine (Sigma).

Plasmid and Strain Generation

For a full description of the plasmids and strains generated for this study see the supplementary information. Attempts to generate a conditional knockout of TgCDPK3 were done in the TATi strain (provided by D. Soldati-Favre, University of Geneva, Switzerland) following the methods previously described (Lourido et al, 2010). All other strains were generated in the Δku80Δhxgprt background, referred to for clarity in this study as $CDPK1^{G}$ (provided by V. Carruthers, University of Michigan, USA). The $CDPK1^{M}$ strain was generated in two stages. First, we introduced, by single site homologous recombination into the endogenous *TgCDPK1* locus, a plasmid carrying the first intron of *TgCDPK1* fused to the cDNA starting at the second exon and ending with a c-terminal c-myc tag. This plasmid contained both a mutation methionine gatekeeper and an hxgprt cassette. Negative selection with 6-thioxanthine was used to remove the sequences between the c-myc tag and the endogenous 3'-UTR of *TgCDPK1*, by double homologous recombination with a construct bearing homology to both sites. The latter strain was named $CDPK1^{M}$, lacked

HXGPRT, and contained the mutant version of TgCDPK1 between the endogenous 5'- and 3'- UTRs. The CDPK1^{M} strain was transfected with a vector containing the second intron of *TgCDPK3* fused to the cDNA starting at the third exon and ending with a c-terminal Ty-tag. Parasites were selected with mycophenolic acid and xanthine for *HXGPRT* present in the vector. Clones were screened by PCR for homologous recombination at the second intron such that they carried the Ty-tagged copy of *TgCDPK3*, downstream of which the endogenous allele was left without a promoter or the fist two exons, and was therefore silent. The alleles were genotyped as coding for a methionine or a glycine at the gatekeeper position by allele specific PCR. Clonal lines were maintained without selection, and no reversion to the wildtype locus was observed during the course of the study.

Immunofluorescence Microscopy

Immunofluorescence staining was performed as described previously (Starnes et al, 2006) following permeabilization with 0.1% saponin (Sigma) with rabbit anti-HA9 (Invitrogen) and mouse anti-SAG1 (mAb DG52), followed by Alexa564-goat anti-rabbit IgG (Invitrogen) and Cy5-goat anti-mouse IgG (Jackson). Images were collected on a Zeiss LSM 510 confocal microscope and analyzed using the LSM 510 Examiner software.

Sequence Alignment

The published alignment of the *T. gondii* active kinases (Peixoto et al, 2010) was used to identify the gatekeeper residues based on the position of subdomain V (Hanks & Hunter, 1995) and the previously identified gatekeeper residue of TgCDPK1 (Lourido et al, 2010).

Protein Purification

Full-length *TgCDPK1* and *TgCDPK3* were PCR amplified from a *T. gondii* RH cDNA library generated using the SMART cDNA synthesis kit (Clontech). The primers used (*TgCDPK1*: 5'-GCGCATATGATGGGGCAGCAGGAAAGCAC and 5'-GCGCTCGAGG-TTTCCGCAGAGCTTCAAGAGC; *TgCDPK3*: 5'-GCGCATATGATGGGGTGCGTCCA-CTCCAAG and 5'-GCGGCGGCCGCGTGCTTCACTTTGACGTCGCAG) contained restriction sites that were used to directionally clone the PCR product, NdeI to XhoI or NotI, into the pET-22b(+) vector, in frame with a C-terminal hexahistidine tag. Mutation of the gatekeeper codon, corresponding to $G^{128}M$ for TgCDPK1 and $M^{153}G$ for TgCDPK3, was achieved using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies), with specific primers designed according to manufacturer instructions. Plasmids were transformed into BL21(DE3)V2RpAcYc-LIC+LamP *Escherichia coli*, as described previously (Wernimont et al, 2010). Following overnight growth in Terrific Broth at 37°C, cells were cooled to 15°C, induced by addition of 1 mM isopropylthio-b-D-galactopyranoside, and returned to culture overnight. Cells were lysed in CellLyticB solution (Sigma), and proteins purified using HIS-select Nickel Affinity Gel following manufacturers instructions (Sigma). Purified proteins were dialyzed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.125% Chelex 100) and stored in 20% glycerol at -20°C. Protein purity and concentration were determined by SDS-PAGE followed by staining with SYPRO Ruby (Invitrogen).

Kinase Assays

Kinase assays were conducted using a peptide-based ELISA. Syntide-2 peptide (10 mg/ml; Calbiochem) was used to coat 96-well plates by overnight incubation in carbonate coating buffer (pH 9.6) at 4°C. Following washing in Tris-tween (50 mM Tris-HCl, pH

7.5, 0.2% Tween20), plates were blocked with 3% BSA in Tris-tween for 2 hr at room temperature, and all further washes were done with Tris-tween. Kinase reactions were conducted at 30°C for 20 min in kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM $CaCl_2$, 0.1 mM EGTA, 0.005% Tween20) containing appropriate amounts of ATP (K_m for each enzyme) and enzyme dilutions (see below). Phosphorylation was detected with mAb MS-6E6 (MBL), followed by peroxidase-conjugated goat-anti-mouse IgG, developed with the substrate 3,3',5,5'-tetramethylbenzidine and detected by absorbance at 450 nm.

Each kinase preparation was individually tested in the assay to establish its half-maximal activity from a dose-response curve plotted in Prism (Graph-Pad). The K_m for ATP was determined for each enzyme tested at its half-maximum, by serial dilution of ATP and plotting in Prism (Graph-Pad). The sensitivity of each enzyme to 3-MB-PP1 was tested at its individual half-maximal activity and K_m for ATP. Triplicate samples were run for all assays. Data were analyzed using Prism (GraphPad) to determine IC_{50} values by plotting normalized, log-transformed data (x-axis), using non-linear regression analysis as a sigmoidal dose-response curve with variable slope.

Western Blotting

Lysates from 1×10^7 parasites per lane were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with rabbit anti-TgALD1, mouse anti-c-myc (Santa Cruz Biotechnology, mouse anti-Ty (mAb BB2) (Bastin et al, 1996), or mouse anti-MIC2 (mAb 6D10). The signal was detected using IRDye 680CW conjugated donkey anti-rabbit IgG (LI-COR Biosciences) and IRDye 800CW conjugated goat anti-mouse IgG (LI-COR Biosciences) on the Odyssey infrared imager (LI-COR Biosciences). Images were processed and analyzed using the Odyssey infrared imaging system software.

Invasion Assays

Invasion was measured as previously described (Huynh et al, 2003). Briefly, parasites harvested in invasion medium (DMEM containing 20 mM HEPES pH 7.5, supplemented with 3% FBS) were incubated in 5 mM 3-MB-PP1 or media containing an equivalent amount of vehicle (DMSO), for 20 min at 37°C, before invasion. Subconfluent HFF monolayers in 24-well plates were infected with the treated parasites (5 x 10^6 per well) and allowed to invade for 20 min. Monolayers were then fixed and stained to distinguish intracellular from extracellular parasites. Samples were performed in triplicate for each experiment and parasite numbers per field were normalized to host-cell nuclei.

Videomicroscopy of Egress and Parasitophorous Vacuole Permeabilization

Egress and PVM permeabilization were analyzed by videomicroscopy as described previously (Håkansson et al, 1999). Sample dishes were allowed to equilibrate for 5 min on the heated stage before the addition of either 2 mM A23187 (EMD) or 0.5 mM Zaprinast (EMD). Vacuoles were imaged for up to 10 min after stimulant addition. To quantify vacuole permeabilization, parasites were transfected with p30-DsRed (Kafsack et al, 2009) (using a plasmid provided by F. Dzierszinski, McGill University, Canada), allowed to infect HFF monolayers, and imaged 24 h post infection. The fluorescence intensity within a 6 m-diameter circular region within each vacuole was measured using the Openlab software. The values for each vacuole were normalized against the starting (100%) values for that particular vacuole. When noted, 5 mM 3-MB-PP1was added 20 min prior to imaging.

Gliding Assays

Gliding was monitored by videomicroscopy as described above. Parasites were harvested in intracellular buffer (5 mM NaCl, 142 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA, 5.6 mM glucose, 25 mM HEPES, pH 7.2 adjusted with KOH) containing 5 mM 3-MB-PP1,

and allowed to settle onto glass chamber slides (Lab-Tek). The supernatant was carefully removed and replaced again with intracellular buffer containing 5 mM 3-MB-PP1 and the dishes were kept at 37°C for up to one hour. Prior to imaging the media in each dish was exchanged for extracellular buffer (141.8 mM NaCl, 5.8 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 25 mM HEPES, pH 7.2 adjusted with NaOH) containing 5 mM 3-MB-PP1, the dish was allowed to equilibrate 4 min on the heated stage, and imaged 4 min, at 1 frame per sec. Images were collected and analyzed with Openlab v. 4.1 (Improvision). Three movies were taken for each strain on a given experiment. The numbers of parasites performing each type of motility were normalized to the total number of parasites in each movie.

Egress Assays

Egress was quantified by measuring lactate dehydrogenase (LDH) release from host cells as a consequence of rupture. In a 96-well format, HFF monolayers were infected with 5 x 10^5 parasites per well. 24 h post infection, monolayers were washed once with mammalian Ringer's buffer supplemented with 1% FBS, and then incubated 20 min in 50 l of the same buffer containing the specified amount of inhibitor or vehicle control (DMSO). To induce egress, 50 l of the same solution was added to each well, but additionally containing either 4 mM A23187 or 0.5 mM Zaprinast, unless otherwise noted. Supernatants were collected and LDH measured using the CytoTox 96 assay (Promega) according to manufacturer's instructions. Values were normalized to total lysis (100%) and uninfected (0%). None of the compounds tested caused significant LDH release in uninfected cells. Samples were assayed in triplicate for each experiment. Data were analyzed using Prism (GraphPad) to determine IC_{50} values by plotting normalized, log-transformed data (x-axis), using non-linear regression analysis as a sigmoidal dose-response curve with variable slope.

Secretion Assays and FACS Analysis

Microneme secretion was assayed by monitoring the release of MIC2 into the culture medium, as described previously (Carruthers et al, 1999). Parasites were pretreated for 20 min with 5 mM 3-MB-PP1 or vehicle control (DMSO) at 37°C before stimulation. Secretion was stimulated by treatment for 10 min with 2% ethanol, alone or in the presence of 3% FBS, at 37°C. Parasite lysis was monitored by the release of actin into the medium: this level remained undetectable in all experiments presented. Samples were resolved by SDS-PAGE and western blots were quantified as described above. Following secretion, parasites were stained for surface accumulation of MIC2, as previously described (Buguliskis et al, 2010). FACS data was analyzed using FloJo 7.4 software (Tree Star Inc.), gated on the parasites by forward and side scatter, and used to generate histograms.

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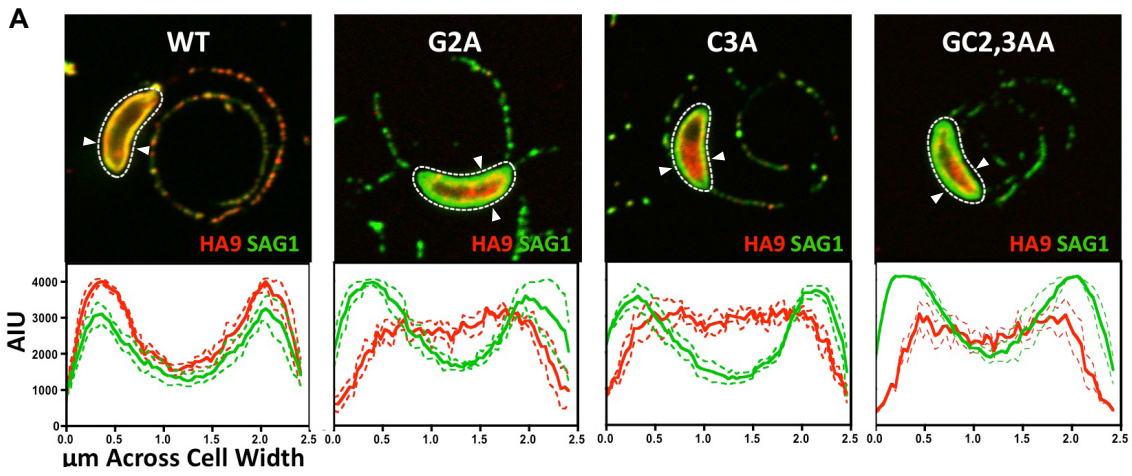

Figure 1. Localization of TgCDPK3 depends on putative acylation sites. Immunofluorescence analysis of parasites expressing different alleles of HA9-tagged TgCDPK3. The region within the dotted line represents an optical slice through the center of the gliding parasite, collapsed onto the slice showing its trail. Graph represents the relative fluorescence intensity across the apical end, marked by the white arrows, of three parasites; Mean ± s.e.m; red, HA9; green, SAG1.

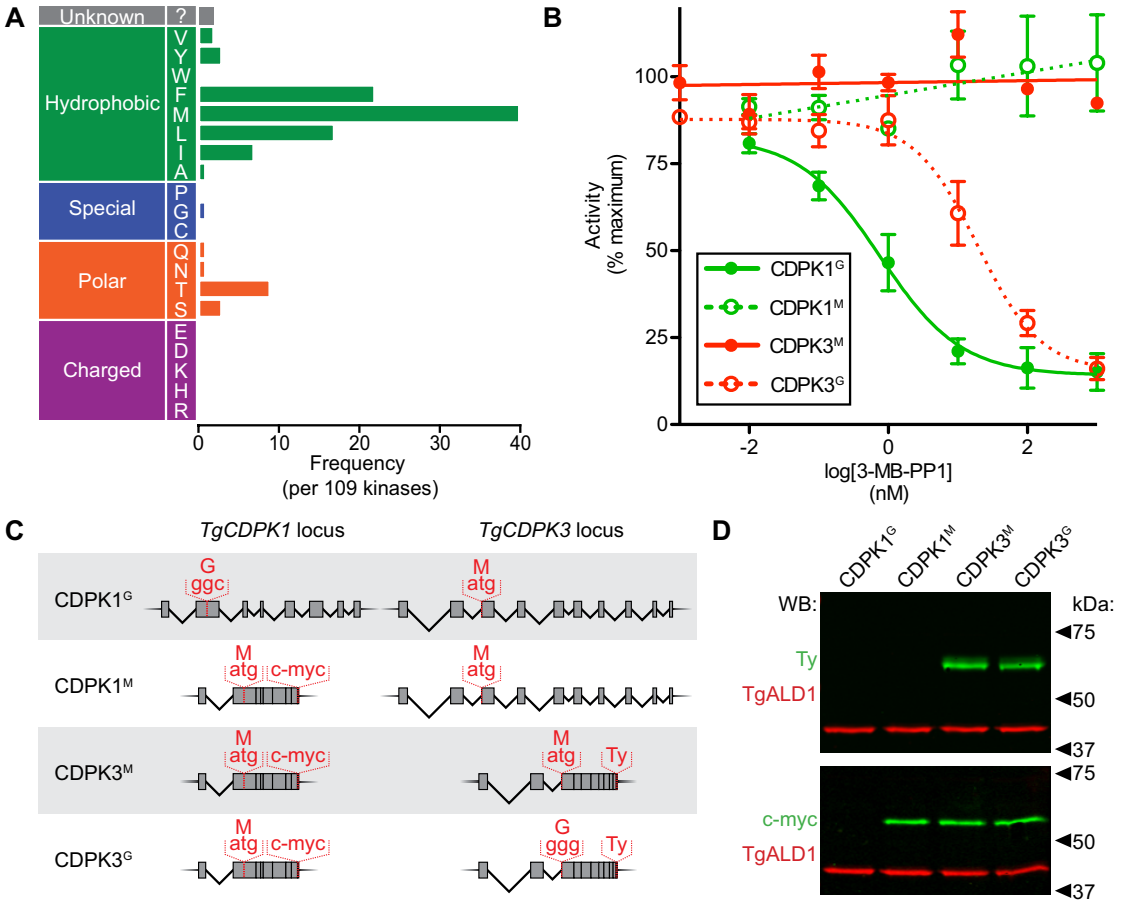

Figure 2. Chemical genetic strategy to inhibit CDPKs *in vitro* and *in vivo*. (**A**) Frequency of gatekeeper residues in the 109 active kinases of *T. gondii*. (**B**) *In vitro* kinase activity against syntide-2. Recombinant CDPK1 or CDPK3 carrying a methionine (M) or glycine (G) gatekeeper were assayed in the presence of different concentrations of 3-MB-PP1. Means ± s.e.m., n = 3 experiments. (**C**) Genotypes of the strains used in this study at the *CDPK1* and *CDPK3* loci. Exons are designated by boxes, and introns by lines. The codon and amino acid for the gatekeeper residue are designated in red, as well as epitope tags introduced in the process of manipulation. (**D**) Immunoblot for the c-myc-tagged *CDPK1*^{M} allele, or the Ty-tagged CDPK3 following allelic replacement. Aldolase was used as a loading control.

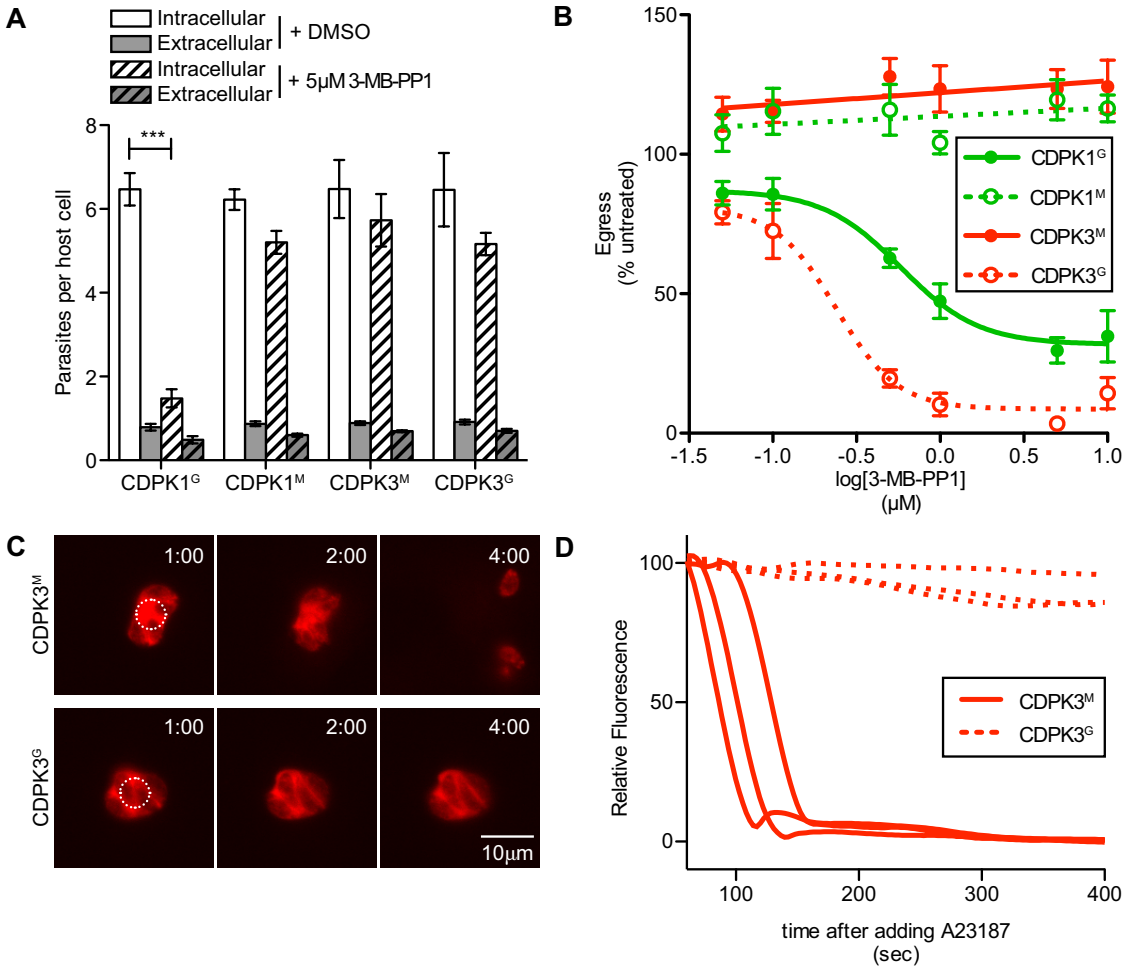

Figure 3. CDPK1 and CDPK3 play different roles in A23187-triggered egress and host cell invasion. (**A**) Invasion of fibroblasts by different strains in the presence of 5 mM 3-MB-PP1 or vehicle alone (DMSO). Extracellular and intracellular parasites were stained differentially and counted relative to the number of host cell nuclei in each field. Student's *t*-test; ***, $P > 0.0005$; means ± s.e.m., n = 3 experiments. (**B**) Egress following 20 min incubation with different concentrations of 3-MB-PP1, and treatment with 2 mM A23187 for 5 min. Egress was measured as a function of lactate dehydrogenase released from host cells, and normalized to the levels resulting from parasites treated with DMSO instead of 3-MB-PP1. Means ± s.e.m., n = 3 experiments. (**C**) A23187-induced vacuole permeabilization detected by vacuolar DsRed leakage monitored by fluorescence videomicroscopy of strains in the presence of 5 mM 3-MB-PP1. The time stamps represent min:sec after the addition of A23187, the circle in the first frame of each movie represents the area quantified for D. (**D**) Relative fluorescence in a circular area with a diameter of 6mM, within the vacuole. The three lines of each genotype, represent measurements from three independent experiments.

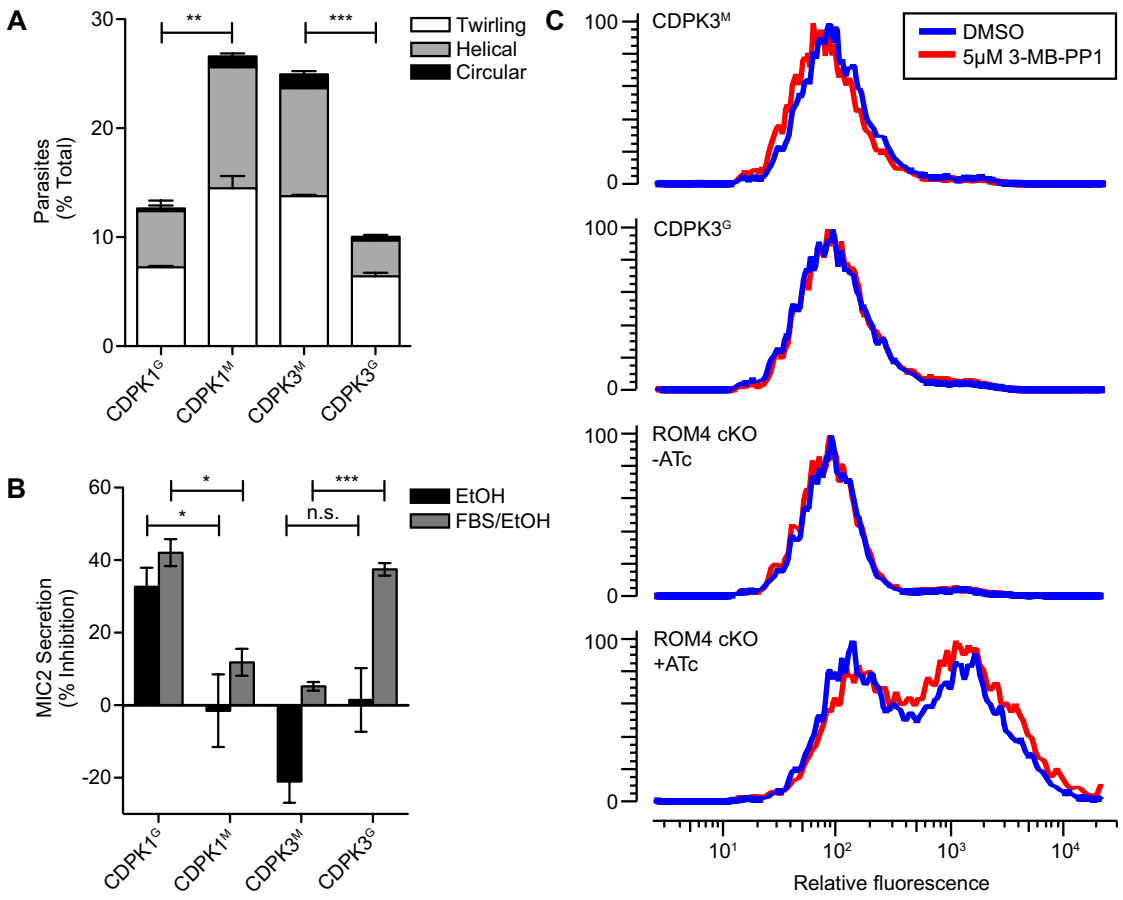

Figure 4. Gliding and microneme secretion following inhibition of CDPK1 or CDPK3. (**A**) Types of gliding motility recorded over 4 min for each strain in the presence of 5 mM 3-MB-PP1. Student's t-test; ***, $P < 0.0005$; **, $P < 0.005$; means ± s.e.m., n = 3 experiments, corresponding to 2-3 videos each. (**B**) Effect of 5 mM 3-MB-PP1 on MIC2 secretion in response to ethanol (EtOH) alone or in combination with FBS (EtOH/FBS). Student's *t*-test; ***, $P < 0.0005$; *, $P < 0.05$; n.s., $P > 0.05$; means ± s.e.m., n = 3 experiments. (**C**) Surface staining for MIC2 following stimulation of secretion by EtOH/FBS in the presence of 5 mM 3-MB-PP1 or vehicle alone (DMSO). The ROM4 cKO was incubated with ATc where noted, and used as a control for the surface accumulation of MIC2. The histogram was generated for the intensity of surface staining of different strains in a representative FACS experiment.

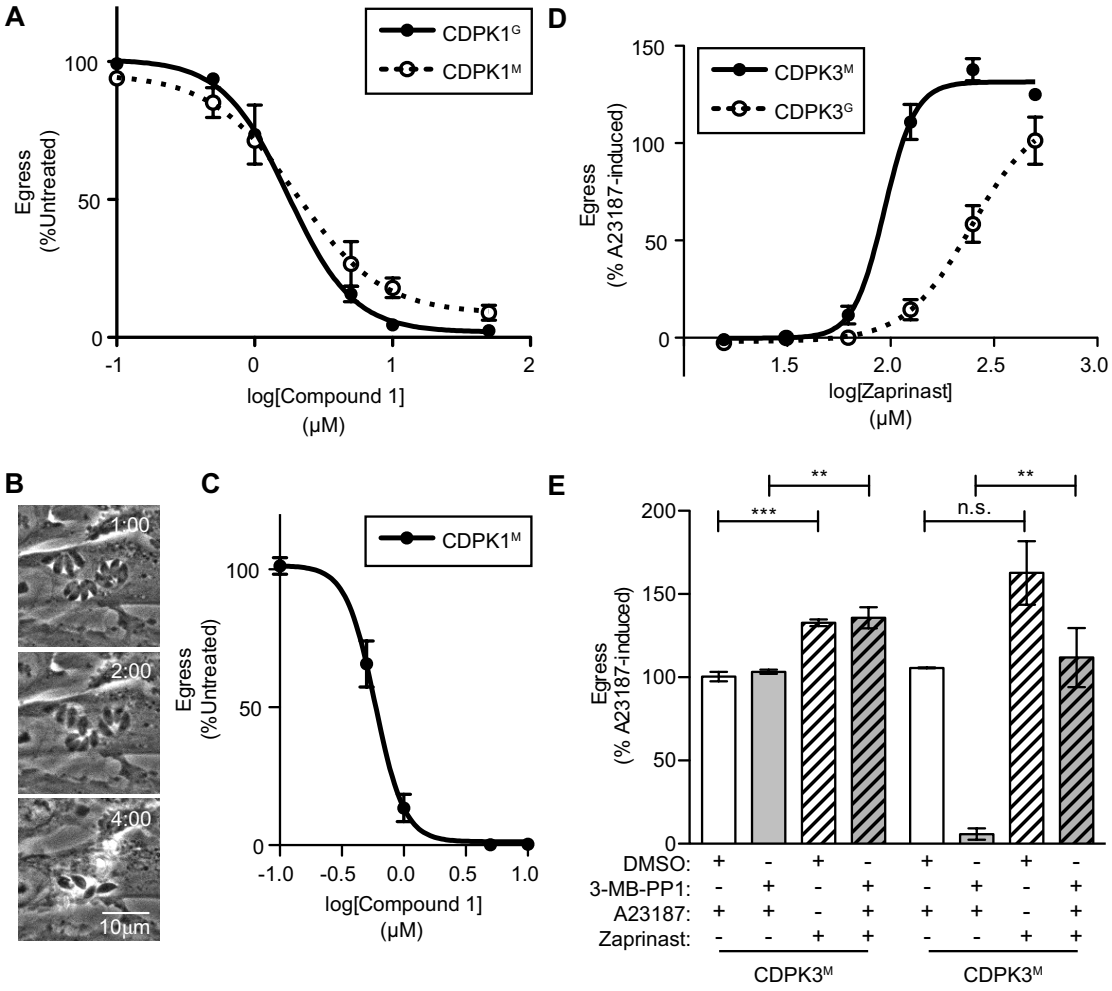

Figure 5. PKG activation compensates for the inhibition of CDPK3 during egress. (**A**) Egress following 20 min incubation with different concentrations of Compound 1, and treatment with 2 mM A23187 for 5 min. Egress was measured as a function of lactate dehydrogenase released from host cells, and normalized to the levels resulting from parasites treated with DMSO instead of Compound 1. Means ± s.e.m., n=3 experiments. (**B**) Strain CDPK3^{M} stimulated by 0.5mM Zaprinast. Egress monitored by videomicroscopy. The time stamps represent min:sec after the addition of Zaprinast. (**C**) Egress following 20 min incubation with different concentrations of Compound 1, and treatment with 0.5 mM Zaparinast for 20 min. Means ± s.e.m., n = 3 experiments. (**D**) Egress triggered by different Zaparinast concentrations after treating strains for 20 min with 5 mM 3-MB-PP1. Egress was measured as described above. Means ± s.e.m., n=3 experiments. (**E**) Comparison of egress following treatment for 20 min with or without 5 mM 3-MB-PP1, and triggered for 20 min with 2 mM A23187 and 0.5 mM Zaparianast alone or in combination. Student's *t*-test; ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$; n.s., $P > 0.05$; means ± s.e.m., n=3 experiments.

SUPPLEMENTARY MATERIALS AND METHODS

Generation of plasmid constructs

To generate the regulatable expression vector for *TgCDPK3,* the cDNA of *TgCDPK3* was amplified from a *T. gondii* RH library, generated using the SMART cDNA synthesis kit (Clontech), with primers 1 and 2 (Supplementary Table 1). The PCR product was digested and cloned directionally with EcoRI/PacI into pS1mycMyoA (Meissner et al, 2001) to make *pTetO7SAG1/TgCDPK3-HA*. The *TUB1/CAT/SAG1* cassette was amplified from pT/230 (Soldati & Boothroyd, 1995) with primers 3 and 4 (Supplementary Table 1), and cloned into the XbaI site of *pTetO7SAG1/TgCDPK3-HA* to make *pTetO7SAG1/TgCDPK3-HA/CAT*. Mutation of the putatively acylated residues in this construct, G^2A and C^3A, alone or in combination, were performed using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies), with specific primers designed according to manufacturer's instructions

To generate the knockout construct for *TgCDPK3*, a tandem yellow fluorescent protein (YFP) driven by the *TUB1* promoter was cloned from *pKOAld:YFP* (Starnes et al, 2009) into the SacI site of *pSAG1/ble* (Messina et al, 1995) making *pSAG1/ble*/YFP. Approximately 1.5 kb from each of the sequences flanking the coding region of *TgCDPK3* were amplified from *T. gondii* RH genomic DNA and introduced into *pSAG1/ble/YFP*. Sequentially, the *5'-UTR*, amplified with primers 5 and 6 (Supplementary Table 1), and the *3'-UTR*, amplified with primers 7 and 8, were cloned directionally into *pSAG1/ble/YFP* using PacI/SacII, and ApaI/HinDIII, respectively, to generate *pTgCDPK3-KO.*

The construct for the allelic replacement of *TgCDPK1* was made in a single step as follows. *pLIC-YFP-HXGPRT* (provided by V. Carruthers, University of Michigan, USA) was digested with PacI/AscI to remove *YFP* and introduce two fragments: (*i*.) the partial genomic coding region of *TgCDPK1* amplified with primers 9 and 10 (Supplementary Table 1) digested with PacI/EcoRI and (*ii*.) the partial c-myc tagged cDNA from the

previously published complementing vector *G^{128}M* (Lourido et al, 2010) amplified with primers 11 and 12 (Supplementary Table 1), and digested with EcoRI/AscI. The resulting plasmid, named *pTgCDPK1-AR-HXGPRT*, contained the first intron of *TgCDPK1* fused to the cDNA, starting at the second exon and ending with a C-terminal c-myc tag, and containing mutation G^{128}M at the gatekeeper position.

The previous construct was modified to remove *HXGPRT* from the *TgCDPK1* locus following allelic replacement. *pTgCDPK1-AR-HXGPRT* was digested with AscI/XbaI to remove the *HXGPRT* cassette and introduce, into the same site, the *TgCDPK1 3'-UTR* amplified with primers 13 and 14 from genomic DNA. The resulting construct was named *pTgCDPK1-AR-3'UTR*.

The construct for the allelic replacement of *TgCDPK3* was generated similarly to that of *TgCDPK1*. *pLIC-YFP-HXGPRT* was digested with PacI/AscI to remove *YFP* and introduce two fragments: (*i*.) the partial genomic coding sequence of *TgCDPK3* amplified with primers 15 and 16 (Supplementary Table 1), and digested with PacI/XhoI and (*ii*.) the partial c-myc tagged cDNA, from the RH library, amplified with primers 17 and 18 (Supplementary Table 1), and digested with XhoI/AscI. The gatekeeper residue in this construct was mutated to code for M^{153}G using the QuikChange II Site-Directed Mutagenesis Kit, with specific primers designed according to manufacturer's instructions. The resulting construct was named *pTgCDPK3-AR-HXGPRT* and contained the second intron of *TgCDPK3* fused to the cDNA starting at the third exon and ending with a c-terminal Ty-tag.

Generation of parasite strains

Attempts to generate a conditional knockout of *TgCDPK3* were done in the TATi strain by first introducing a conditional allele and then attempting to knockout the endogenous allele by double homologous recombination. TATi was transfected with *pTetO7SAG1/*

TgCDPK3-HA/CAT and parasites were selected with chloramphenicol (Kim et al, 1993). Clonal lines were derived by limiting dilution and checked by immunofluorescence microscopy and western blot for the presence of HA9-tagged TgCDPK3. Strains carrying the mutant alleles of TgCDPK3 were generated in the same fashion, and all expressed the HA9-tagged alleles to the same level, as determined by western blot using TgALD1 as a loading control. Attempts to knock out the endogenous *TgCDPK3* were performed using the merodiploid strain expressing the wild type regulatable allele. The knockout construct, *pTgCDPK3-KO* was linearized with ApaI and transfected into the merodiploid strain. Stable pools were selected through two rounds of phleomycin selection (Messina et al, 1995), and YFP-negative parasites were sorted to enrich for potential knockouts. Individual clones were isolated by limiting dilution and screened by PCR to check for loss of the endogenous allele, as determined by using primers against consecutive exons and the intervening intron, to distinguish between the endogenous and regulatable genes.

All other strains were generated in the Δku80Δhxgprt background, referred to for clarity in this study as CDPK1^{G} (provided by V. Carruthers, University of Michigan, USA). The CDPK1^{M} strain was generated in two stages. First, we transfected CDPK1^{G} with *pTgCDPK1-AR-HXGPRT* that had been linearized with BsiWI, and selected parasites with mycophenolic acid and xanthine before isolating clones by limiting dilution. Individual clones were checked by PCR in reactions specific to the allelic replacement, with primers 19 and 20 (Supplementary Table 1). It should be noted that primer 19 sits outside the region cloned into *pTgCDPK1-AR-HXGPRT* and under the reaction conditions used primer 20 only functioned in the presence of the gatekeeper mutation. Therefore, PCR reactions were only positive for single site homologous recombination events that led to a mutation of the *TgCDPK1* gatekeeper. Individual clones were additionally checked by western blot for expression of the c-myc tagged TgCDPK1. In order to remove the *HXGPRT* cassette and the portion of the endogenous allele that had been left without a promoter or the first

exon, downstream of the allelic replacement, the strain was transfected with *pTgCDPK1-AR-3'UTR*, which had been linearized with PacI/XbaI. Parasites were selected with 6-thioxanthine (Fox et al, 2009) and clones were isolated by limiting dilution. Individual clones were screened for the loss of *HXGPRT*, with primers 21 and 22 (Supplementary Table 1), and for the correct arrangement of the new allele with the endogenous *3'-UTR* of *TgCDPK1*. The latter strain was named CDPK1^{M}, lacked *HXGPRT*, and contained the mutant version of *TgCDPK1* between the endogenous *5'-UTR* and *3'-UTR*.

To manipulate the *TgCDPK3* locus, the CDPK1^{M} strain was transfected with *pTgCDPK3-AR-HXGPRT*, which had been linearized with SphI. Parasites were selected with mycophenolic acid and xanthine before isolating clones by limiting dilution. Individual clones were screened by PCR for homologous recombination leading to Ty-tagged *TgCDPK3* bearing either the wild type methionine gatekeeper, and therefore positive for the reaction with primers 25 and 26 (Supplementary Table 1), or the gatekeeper mutated to a glycine, and positive for the reaction with primers 25 and 27 (Supplementary Table 1). The PCR reaction conditions were optimized with known templates, to preclude cross-reactivity, and ensure correct diagnosis of the allele identity. Strains were also checked by western blot for presence of the Ty-tagged TgCDPK3, using TgALD1 as a control. The strain carrying the methionine gatekeeper was named CDPK3^{M}, and that carrying the mutant glycine gatekeeper was named CDPK3^{G}.

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	Number	Sequence (5' to 3')
pTet07SAG1_TgCDPK3-HA9_cat	1	GCGGAATTCCCTTTTTCGACAAAATGGGGTGCGTCCACTC-CAAG
	2	GCGTTAATTAATTACGCATAGTCAGGAACATCGTATGGG-TAGTGCTTCACTTTGACGTCGCAG
	3	GCGTCTAGACGGGCCCCCCCTCGAGGTCGAC
	4	CGGCCGCTCTAGAACTAGTGGAT
pTgCDPK3-KO	5	GCGTTAATTAAGTCCGTTCGAAGCGTCTTGTGTG
	6	GCGCCGCGGCCTTTGATTTCCTTCAGAATAC
	7	GCGGGGCCCCGCCACTGCTCCCAAACAACGCG
	8	GCGAAGCTTGGTGACTTTGGCGTGCGCTGGTC
pTgCDPK1-AR-HXGPRT	9	GCGTTAATTAACTCGCATGCATGTCGCTGGTC
	10	CGCCGACGAGGTAGAAGTAG
	11	TACAAGGGACAGCGGGTGTTG
	12	TATGGCGCGCCTTACAGATCCTCTTCAGAGATGAG
pTgCDPK1-AR-3'UTR	13	TATGGCGCGCCAAGCTTGCACTCTCCCTGTGCG
	14	GCGTCTAGACCGCGCTCATCTCAGTTTCCTGG
pTgCDPK3-AR-HXGPRT	15	GCGTTAATTAAGTTGTGAAGAACCTTCTGGCG
	16	GAGCGTCCCGGCTCTTCGAC
	17	GCGACCTGAAGCCCGAAAAC
	18	TATGGCGCGCCTTAATCGAGCGGGTCCTGGTTCGTGTG-GACCTCGTGCTTCACTTTGACGTCGC
TgCDPK1 allelic replacement	19	AGGCGTTGCGGTCGCTGTCTC
	20	GCCTCCCGTGTACACTTCCAT
HXGPRT	21	TTTCGCTTTAGTAGTGCGTTTCTG
	22	GGGCTATGCAGGGTTTACTTCTCG
TgCDPK1 locus cleanup	23	TCATCTCTGAAGAGGATCTG
	24	CGGCGTCTTGTGTCGTTTATCAG
TgCDPK3 allelic replacement	25	GTTTCCTCGACGTGTGTTCAG
	26	CCTCCTCGGTACACCTCCAT
	27	CCTCCTCGGTACACCTCCCC

Supplementary Table 1. Primers used in this study.

CHAPTER IV

Identification of Proteins Phosphorylated by Calcium-Dependent Protein Kinase 1 in *Toxoplasma*

PREFACE

Work presented in this chapter was conducted by Sebastian Lourido, D. Kesley Robertson, and Wandy Beatty. SL designed and performed the majority of experiments, analyzed the data, and generated the figures. WB prepared the samples for electron microscopy and DKR collected the images of apical sections. The first draft of this chapter was written by SL. Comments from L. David Sibley were incorporated into the final version presented here. Portions of this manuscript will be submitted for publication pending further experiments.

ABSTRACT

Apicomplexan parasites rely on gliding motility to invade host cells and establish a site of intracellular replication. Cycles of invasion and replication lead to the tissue destruction associated with pathology making host-cell invasion central to both parasite survival and pathogenesis. Gliding motility is dependent on the apical secretion of adhesins, followed by their posterior translocation, propelling the parasite forward. An increase in intracellular calcium is known to regulate secretion of adhesins, however the molecular mechanisms underlying this regulation remain unknown. We have previously demonstrated that in *Toxoplasma gondii*, calcium-dependent protein kinase 1 (TgCDPK1) is required for the calcium-regulated secretion of adhesins. In the present study, we use a chemical-genetic approach to identify targets of TgCDPK1 phosphorylation and elucidate novel components regulating microneme secretion. Our studies reveal that a dynamin-related protein previously associated with microneme biogenesis is phosphorylated during microneme secretion in a TgCDPK1-dependent manner. We observe that loss of TgCDPK1 leads to an accumulation micronemes at the apical end, and propose a model for the role of a dynamin-related protein in exocytosis.

INTRODUCTION

Host cell invasion by apicomplexan parasites relies on gliding motility, which uses posterior translocation of apically secreted adhesins to generate substrate-dependent forward movement in the absence of cell deformation. Adhesins are translocated via actomyosin motor complexes anchored in a network of flattened cisternae under the plasma membrane. These cisternae are common to all alveolates, but in apicomplexans they are flattened and fused in an arrangement called the inner membrane complex (IMC), which subtends nearly the entire plasmalemma, except at the poles (Morrissette *et al*, 1997). Adhesins are released from specialized organelles called micronemes, small elongated vesicles associated with the apical end of the parasite. In ultrastructure studies of *Toxoplasma gondii* micronemes appear to be secreted through the conoid (Carruthers & Sibley, 1999), which is an open basket arrangement of tubulin that sits in the apical opening of the IMC (Hu *et al*, 2002). Although the conoid itself is missing from some invasive stages of other apicomplexans, the polar secretion of micronemes is a conserved feature. Activation of secretion and motility by a surge of intracellular calcium has been shown to occur in *T. gondii* (Carruthers & Sibley, 1999), *Cryptosporidium parvum* (Chen *et al*, 2004), and *Plasmodium falciparum* (Singh *et al*, 2010). Various stimuli can trigger motility including host cell rupture (Moudy *et al*, 2001), accumulation of abscisic acid in the environment (Nagamune *et al*, 2008), and host cell contact (Carruthers & Sibley, 1997). It is not clear how these different stimuli are sensed, but their effects are blocked by cytosolic calcium chelators, and once motility is initiated calcium levels can be seen to oscillate by Fluo-4 imaging (Lovett & Sibley, 2003).

In animals, calcium also plays a role in regulated secretion, including the release of insulin from pancreatic cells (Easom, 1999), histamine from mast cells (Sim *et al*, 2006), and neurotransmitters from neurons (Barclay *et al*, 2005). In these cells, many of the proteins that respond to calcium are known, including those that prevent or promote vesicle fusion, and enzymes that in turn modulate other proteins (Barclay *et al*, 2005). A number of C2-

domain containing proteins are known to participate in synaptic vesicle fusion, including DOC2 (Barclay *et al*, 2005). By homology, a similar function has been attributed to a DOC2 protein in apicomplexans, which was originally identified in a chemically-induced temperature sensitive mutant screen, and shown to be required for microneme secretion (Farrell *et al*, 2012).

Calcium-regulated kinases of animals, are also known to play important roles in regulated exocytosis, including protein kinase C (PKC) and calcium/calmoduling dependent protein kinase (CaMK) (Sim *et al*, 2006; Barclay *et al*, 2005). Apicomplexan genomes lack an identifiable PKC, but possess a large family of calcium-dependent protein kinases, related to CaMKs, but activated directly by a calmodulin domain fused to their kinase domain (Billker *et al*, 2009). In *Plasmodium spp.* many of these kinases are dispensable for growth in erythrocytes and have been knocked out leading to defects in later developmental stages including male gamete exflagellation (Ishino *et al*, 2006), ookinete motility (Siden-Kiamos *et al*, 2006), and sporozoite recognition of hepatocytes (Coppi *et al*, 2007). More recently, conditional expression of a CDPK in *P. falciparum* demonstrated its role in egress from erythrocytes (Dvorin *et al*, 2010). Taken together, these studies demonstrate that these kinases play a central role in parasite biology, although the precise molecular functions for these kinases have yet to be established. Using a conditional knockout, we have previously demonstrated that *T. gondii* calcium-dependent protein kinase 1 (TgCDPK1) is required for the secretion of micronemes, suggesting that the participation of kinases in regulated secretion might also be a conserved feature of this process (Lourido *et al*, 2010). However, none of the phosphorylated proteins known to regulate secretion in animals, like Rabphilin or synapsing (Barclay *et al*, 2005), appear to be conserved in apicomplexans, based on BLAST searches, and the mechanism for TgCDPK1 regulation of microneme secretion remains unknown.

We and others have exploited the atypical ATP-binding pocket of TgCDPK1 to specifically inhibit this kinase *in vivo* (Lourido *et al*, 2010; Ojo *et al*, 2010; Murphy *et al*, 2010; Johnson *et al*, 2012). Unique among parasite kinases, TgCDPK1 contains a glycine in the ATP-binding pocket, termed the gatekeeper. This glycine extends this normally shallow pocket, enabling the binding of bulky pyrazolo [3,4-d] pyrimidine (PP) derivatives (Ojo *et al*, 2010), and subsequent inhibition of the kinase activity. Mutation of the gatekeeper residue of TgCDPK1 to a methionine prevents inhibition, and has allowed us to demonstrate the specificity of these compounds in parasites (Lourido *et al*, 2010). We have also replaced the endogenous allele of TgCDPK1 with one carrying a methionine gatekeeper to generate a background in which to sensitize other kinases to the PP analogues (Lourido *et al*, submitted; Chapter 3). In the present study, we have adapted a complementary approach, which uses a bulky ATPγS analogue, which can only be used by TgCDPK1, to thiophosphorylate its targets, allowing us to track and isolate them. Various techniques have previously been developed to isolate the thiophosphorylated targets of mammalian kinases (Allen *et al*, 2007; Blethrow *et al*, 2008) and here we adapt them to identify the targets of TgCDPK1. We identify various proteins modified by TgCDPK1 and confirm the phosphorylation of one of them *in vivo*, suggesting a role in the regulation of microneme secretion. Furthermore, this study serves to emphasize the potential of chemical genetics in enabling us to investigate both the phenotypes and cellular pathways regulated by these essential kinases.

RESULTS

TgCDPK1 Regulates Conoid Extrusion and Density of Apical Micronemes

We previously generated a conditional knockout (cKO) of TgCDPK1 where the endogenous allele was deleted in the presence of a tetracycline-regulatable allele (Lourido *et al*, 2010). Growth in the presence of ATc strongly inhibits the ability of these parasites to secrete micronemes, affecting many of the processes known to be dependent on microneme proteins, including lysis of the parasitophorous vacuole, motility and host-cell invasion (Lourido *et al*, 2010). To further investigate this phenotype at an ultra-structural level, we stimulated microneme secretion by incubating parasites with 3% fetal bovine serum (FBS) and 2% ethanol (EtOH), and fixed and processed them for conventional ultrastructural analysis. Analysis of the apical ends of unstimulated parasites revealed that the tip of the conoid was at the same level or slightly below that of the apical end of the IMC (top panel; Figure 1A.). However, upon stimulation of microneme secretion, nearly half of the parasites showed an extruded conformation where the conoid protruded beyond the apical end of the IMC (bottom panel; Figure 1A). Conoid extrusion has been shown to depend on intracellular calcium (Mondragón & Frixione, 1996), and the phenotype we observed is therefore consistent with the surge in intracellular calcium known to be triggered by EtOH treatment (Carruthers *et al*, 1999b). Interestingly, the conoid never protruded beyond the point where its posterior end was flush with the apical end of the IMC, suggesting that the extreme apical extension observed with similar treatments, such as ethanol under light microscopy must represent additional elongation of the apical end along with conoid extrusion (Mondragón & Frixione, 1996; del Carmen *et al*, 2009). To determine whether conoid extrusion could occur in the absence of TgCDPK1, we grew the cKO strain in the presence or absence of ATc 72 h, then treated parasites with either media alone or supplemented with FBS and EtOH. Surprisingly, we did not find any parasites with an extruded conoid when TgCDPK1 was suppressed, although no other gross morphological

changes could be observed when compared to parasites grown in the absence of ATc. To get a quantitative view of the apical ultra-structure we scored samples, in a blinded manner, for conoid extrusion and the number of micronemes in the apical end. As we had qualitatively observed, parasites grown in the presence of ATc were significantly impaired in conoid extrusion in response to stimulation, compared to parasites grown in the presence of ATc (Figure 1B). Quantitative analysis also revealed a previously unappreciated increase in the number of micronemes present in the apical end of parasites lacking TgCDPK1 following stimulation (Figure 1C). This accumulation was specific to stimulated parasites, and was not observed in parasites incubated with media alone, showing that it is not a general consequence of TgCDPK1 loss (Figure 1C).

TgCDPK1 Thiophosphorylates Heterologous Substrates *In Vitro*

Bulky ATP analogues, which only fit in the ATP-binding pockets of kinases like TgCDPK1, have previously been used to specifically label the targets of kinases engineered to bind them (Witucki *et al*, 2002). By replacing the γ-phosphate of these artificial ATP-analogues with a thiophosphate, the targets of a given kinase can be isolated with chemistry specific to the thio group (Allen *et al*, 2007). To determine whether this strategy could be used to study the targets of CDPK1, we incubated recombinant kinases harboring either the wild type glycine or a methionine gatekeeper with dephosphorylated myelin basic protein (dMBP), a heterologous protein that can be phosphorylated by a variety of kinases. As substrates for the phospho-transfer reaction, we added either N6-benzyladenosine-5’-O-[3-thiotriphosphate] (Bn-ATPγS), N6-furfuryladenosine-5'-O-[3- thiotriphosphate] (Fu-ATPγS) or N6-[2-phenylethyl]adenosine-5'-O-[3- thiotriphosphate] (PhEt-ATPγS). Thiophosphorylation of dMBP was detected following alkylation with p-nitrobenzyl mesylate (PNBM) using an antibody that specifically recognizes alkylated thiophosphates (rabbit mAb 51-8). We observed that only Fu-ATPγS was used by wildtype TgCDPK1 to

thiophosphorylated dMBP (Figure 2A.). Although we have previously shown that mutation of the gatekeeper to a methionine preserves normal kinase activity (Lourido *et al*, 2010), the ability of TgCDPK1 to thiophosphorylate dMBP depended on the presence of a glycine gatekeeper ($CDPK1^{G}$) since the mutant kinase ($CDPK1^{M}$) was unable to thiophosphorylate dMBP (Figure 2A). Consistent with the established role of Ca^{2+} in the activation of TgCDPK1, thiophosphorylation of dMBP by $CDPK1^{M}$ was Ca^{2+}-dependent (Figure 2B).

Targets Specifically Thiophosphorylated by TgCDPK1 can be Detected in *T. gondii* Lysates

To determine whether TgCDPK1 could thiophosphorylate targets in the context of parasite lysates, we decided to compare thiophosphorylation in strains carrying either wild type ($CDPK1^{G}$) or the methionine gatekeeper allele ($CDPK1^{M}$). The latter strain was generated by replacing the endogenous allele with one carrying the gatekeeper mutation through homologous recombination (Lourido *et al*, submitted; Chapter 3). Since the mutant strain was generated in the RHΔku80Δhxgprt background (Huynh & Carruthers, 2009), this strain will be used as the comparator wild type (i.e. it harbors $CDPK1^{G}$) in the present study.

Parasite lysates were prepared from each strain and incubated in a reaction buffer containing Fu-ATPγS and either 0 or 10 mM free Ca^{2+}. Lysates were then alkylated with PNMB and proteins resolved by SDS-PAGE, followed by immuno-blotting for either GRA1 as a loading control, or thiophosphorylation with an antibody specific to the alkylated form of the modification (rabbit mAb 51-8). Thiophosphorylated proteins were only detected in lysates from the strain harboring $CDPK1^{G}$ (Figure 3A), and as expected for a CDPK, only in the presence of free Ca^{2+} (Figure 3A).

As a first attempt at isolating the targets of TgCDPK1 from the complex lysate, we used the rabbit mAb 51-8 to immunoprecipitate thiophosphorylated proteins following

alkylation. As a control for non-specific binding, we used the lysates from CDPK1^{M}, as these would also contain any background activity attributable to other kinases. The immunoprecipitated proteins were resolved by SDS-PAGE and stained with SYPRO Ruby (Invitrogen). Although the majority of the observed bands were present in both samples, a number of bands were specifically detected in the CDPK1^{G} sample, potentially representing targets of TgCDPK1 (Figure 3B). Two bands between 100 and 150 kDa were observed by both immunoprecipitation and when probing the entire lysate for thiophosphorylated proteins.

There are two strategies to identify thiophosphorylated proteins using the chemical adduct method we have employed here (Figure 3C). As described above, the monoclonal antibody specific for the modification can be used to immunoprecipitate the proteins, and individual proteins enriched in these samples could be identified by mass spectrometry (MS). Alternatively, the samples can be digested to yield peptides that can be captured covalently with an iodoacetyl resin that immobilizes peptides with free sulfhydryl groups. Thiophosphorylated peptides are then removed from the resin by oxidation, leaving a phosphate group on the residue previously thiophosphorylated. These peptides are then identified, and the position of the thiophosphorylation mapped by MS. Both strategies were performed for the CDPK1^{G} and CDPK1^{M} samples. We restricted our analysis to proteins for which at least two peptides were identified with a probability greater than 90% (Scaffold). Based on these criteria 68 proteins were identified uniquely in the CDPK1^{G} sample, 27 proteins were identified in both samples and only 10 exclusively in the CDPK1^{M} sample in the immunoprecipitation experiment. Fewer proteins overall were identified by the direct peptide capture strategy, but all of them carried phosphorylation of either a serine or a threonine. By the latter method, 18 proteins were identified exclusively in the CDPK1^{G} sample, a single protein was found in both samples, and none were found only in the CDPK1^{M} sample (a complete list of identified proteins can be found in Supplementary Table

1). In total, 6 proteins were identified in both CDPK1^{G} datasets and none of the CDPK1^{M} datasets (Table 1). Although these likely represent only a few of the thiophosphorylated proteins in these lysates, having identified these 6 proteins by two separate methods gave us the confidence to further characterize their thiophosphorylation in lysates and their phosphorylation *in vivo*.

Two Conserved Proteins are Thiophosphorylated by TgCDPK1

Of the six proteins identified as thiophosphorylated exclusively in the CDPK1^{G} samples, only one of them had been previously studied, a dynamin-related protein, DrpB (TGGT1_064650) (Breinich *et al*, 2009). The other five proteins were annotated as hypothetical (ToxoDB) and had no identifiable protein domains. Although the thiophosphorylation experiments were performed in RH, a strain closely related to GT1, one of the proteins was identified by its ME49 gene ID due to its absence from the GT1 annotation (TGME49_005320). A comparison of the two genomes revealed that the GT1 locus contained additional sequence in frame with the predicted ME49 protein. Both predicted proteins contain a central portion of repeats with a periodicity of 28 residues, and the GT1 protein contained an additional three repeats, which might have contributed to its misannotation. Because many of the phosphorylated peptides identified came from these repeats, TGME49_005320 will be called henceforth phosphorylated repeat protein (PRP).

As calcium regulation of microneme secretion is a conserved feature in apicomplexans, we predicted that the targets of TgCDPK1 would also be conserved, at least within the phylum. Other than DrpB, which had clear homologues in all apicomplexan genomes, only PRP was conserved across all apicomplexans (OrthoMCL DB). One PRP homolog was found in each of the other apicomplexan genomes, although only the C-terminal portion of the protein showed significant conservation, for an overall 33.6% average identity (ortholog group OG5_141752; OrthoMCL DB). Despite the phylogenetic

distance of these proteins, all prossessed a series of 28-residue repeats in their C-termini. Because of their conservation among apicomplexan genomes, we decided to generate parasite strains in which we could determine their thiophosphorylation in lysates and their phosphorylation *in vivo*. Additionally, DrpB had been previously reported to be involved in the biogenesis of rhoptries and micronemes, based on the loss of these organelles following expression of a dominant negative mutant (Breinich *et al*, 2009). Although these observations do not match the phenotype of TgCDPK1, it remains possible that DrpB also participates in microneme secretion.

To assess the modification of DrpB and PRP, we cloned each along with 1.5 kb upstream of the translation start site, to mimic endogenous expression. Both coding sequences were Ty-tagged; C-terminally for PRP; and N-terminally for DrpB, according to how it had been tagged in previous studies (Figure 4A; Breinich *et al*, 2009). Both constructs were transfected into the TgCDPK1 cKO and stable integration was selected for with pyrimethamine. Immunofluorescence analysis of the resulting strains showed that PRP was distributed in punctate structures throughout the cell, while DrpB was concentrated in the apical end, as previously reported (Breinich *et al*, 2009).

To determine whether TgCDPK1 was required for the thiophosphorylation of the putative targets, we compared strains grown in the absence of ATc with strains grown in the presence of ATc, to determine the effect of TgCDPK1 expression. Lysates from the different strains were incubated in a reaction buffer containing Fu-ATPγS, and the putative targets were immunoprecipitated with the Ty-tag. To control for non-specific immunoprecipitation, we also included the parental cKO strain, which lacked any Ty-tagged proteins. Although comparable amounts of both DrpB and PRP were immunoprecipitated, irrespective of growth with or without ATc (Figure 4C), robust thiophosphorylation was only detected when strains were grown without ATc. This confirms that both DrpB and

PRP are thiophosphorylated in a TgCDPK1-dependent manner, validating our strategy for identifying thiophosphorylated proteins.

TgCDPK1 Regulates DrpB Phosphorylation During Microneme Secretion *In Vivo*

Based on our finding that TgCDPK1 could thiophosphorylate DrpB in lysates, we sought to determine whether DrpB was indeed phosphorylated *in vivo* in a TgCDPK1-dependent manner. The TgCDPK1 cKO strain carrying the Ty-tagged DrpB was grown in the presence or absence of ATc. Extracellular parasites were then incubated for 1 h in the presence of [^{32}P] orthophosphate in order to radiolabel the cellular ATP pool. Labeled parasites were then incubated for a further 5 min in media alone, or supplemented with FBS and EtOH to trigger microneme secretion. Following stimulation, parasites were placed on ice and lysed in detergent. Equal amounts of DrpB were immunoprecipitated under all conditions, but the amount of radiolabel incorporated into DrpB significantly increased in parasites that had been stimulated to secrete (Figure 5A). In contrast, when TgCDPK1 expression was suppressed by growth in ATc, labeling of DrpB was significantly reduced, regardless of stimulation (Figure 5A). These differences were replicated in three independent experiments, demonstrating that DrpB is phosphorylated in a TgCDPK1-dependent manner, in response to a microneme secretion agonist (Figure 5B). An additional effect of the N-terminal tagging of DrpB was that in all our pull-downs we captured both full-length and what appear to be C-terminal truncations of the protein, which had also been observed in other studies with DrpB and the related DrpA (van Dooren *et al*, 2009; Breinich *et al*, 2009). Our observation that the truncated forms of the protein are neither thiophosphorylated (Figure 4C) nor phosphorylated *in vivo* (Figure 5A), suggests that modification occurs in the C terminus of DrpB, consistent with the location of sites identified by peptide capture (Table 1).

Role of DrpB Phosphorylation in TgCDPK1-Regulated Phenotypes

Despite having determined that DrpB phosphorylation requires TgCDPK1, and is stimulated by secretagogues, we have yet to demonstrate a direct requirement for DrpB in microneme secretion. Having identified the putative phosphorylation sites on the DrpB C-terminus, we sought to express different DrpB mutants under the regulation of the FKBP degradation domain (DD), previously used to express a dominant negative DrpB allele (Breinich *et al*, 2009). Expression of the DD-tagged protein was regulated by the addition of Shld-1, which stabilizes the domain and prevents protein degradation (Herm-Götz *et al*, 2007). We introduced the DD in frame with the Ty-tagged DrpB, under the regulation of its endogenous promoter (DD-Ty-DrpB; Figure 6A). However, the resulting construct was expressed both in the presence and absence of Shld-1 (Figure 6B), a technical limitation that sometimes occurs with this strategy. We then obtained the construct that had been previously used, in which DrpB was tagged with DD followed by GFP and c-Myc, under the regulation of the TUB1 promoter (DD-GFP-DrpB; Figure 6A). Consistent with previous reports, this construct was indeed regulated, and GFP expression was only observed in the presence of Shld-1 (Figure 6B). However, the two constructs showed quite different localizations. While the Ty-tagged construct displayed a similar distribution to Ty-DrpB, and to previously reported localization of DrpB with a specific antibody (Breinich *et al*, 2009), the GFP construct was localized to one or a few puncta within each parasite. It is unclear whether this localization is an artifact of GFP-tagging or overexpression. Hence, we decided to use a different method to regulate DrpB expression.

To minimize the size of the epitope tag, we decided to regulate the expression of Ty-tagged DrpB with a previously described tetracycline-inducible promoter (TetON), in which multiple TetO sequences were introduced into the *T. gondii* RPS31 promoter (van Poppel *et al*, 2006). In addition to full-length DrpB, we expressed two C-terminal truncations of the tail domain following the DrpB GED: 117 and 121 amino acid truncations.

These truncations were chosen because of the following features: (*i*.) the predicted phosphorylation sites are located in this region, (*ii*.) the extension is unique to DrpB and its orthologues in other apicomplexans, (*iii*.) it is enriched in serines and threonines, and (*iv*.) it is absent from other apicomplexan dynamin-related proteins. All three alleles were stably integrated, and expressed DrpB to similar levels when grown in the presence of ATc (Figure 6C). We performed a number of experiments to determine whether expression of the truncated forms of DrpB was detrimental to the parasite. However, as demonstrated by the lytic assay, expression of either mutant allele had no effect on host cell lysis, compared to parasites expressing wildtype DrpB (Figure 6D). These results demonstrate that loss of the C-terminal tail does not produce a dominant negative allele of DrpB. Further experiments will be needed to address the function of the tail domain and of the specific phosphorylation sites identified, in a context where these are the only alleles present in the cell.

DISCUSSION

We previously demonstrated that microneme secretion depends on the activity of TgCDPK1 (Lourido *et al*, 2010). In the present study we explore the pathways regulated by TgCDPK1 first by analyzing the phenotype of the TgCDPK1 cKO at a subcellular level and next by investigating the targets of phosphorylation. We observed that conoid extrusion was blocked in the absence of TgCDPK1, and this was correlated with an increase in microneme density at the apical end, in response to secretagogues. To identify the cellular targets of TgCDPK1, we made use of a chemical-genetic strategy that enabled specific thiophosphorylation of TgCDPK1 targets. Two of the targets identified have homologues in other apicomplexans, and we could demonstrate that they were indeed thiophosphorylated by TgCDPK1. One of these substrates, DrpB had been previously implicated in the biogenesis of secretory organelles. Using radiolabeled parasites we were able to show that DrpB is phosphorylated *in vivo* in a TgCDPK1-dependent manner, and its phosphorylation increases when microneme secretion is triggered. Although we have been unable to determine the functional consequences of DrpB phosphorylation, our results validate the use of this chemical-genetic approach to identify the targets of TgCDPK1 and other *T. gondii* kinases.

Its been previously observed that treatment of parasites with calcium ionophore induces conoid extrusion (Mondragón & Frixione, 1996), although the propose of this process remains enigmatic. Looking to better understand the regulation of microneme secretion, we discovered that conoid extrusion also failed to occur in parasites lacking TgCDPK1. By quantifying the number of micronemes in close proximity to the apical end, we also observed a significant accumulation of these vesicles in the absence of TgCDPK1 when secretion was stimulated. Together, these observations suggest that conoid extrusion and microneme secretion are functionally connected, and dependent on the calcium activation of TgCDPK1. Whether the link between these two processes lies in

their common requirement for TgCDPK1 or in that one requires the other, may be resolved by further insight into the cellular targets of TgCDPK1.

Phosphorylation is known to mediate calcium-regulated secretion is animals. In neurons, calcium/calmodulin-dependent protein kinases (CaMKs) phosphorylate synapsins to remove them from secretory vesicles and enable neurotransmitter release (Barclay *et al*, 2005). However despite the ubiquity of calcium-regulated exocytosis in organisms ranging from plants (Schapire *et al*, 2009) to ciliates (Plattner *et al*, 2012), proteins known to be phosphorylated in this process are generally not conserved. Therefore it is not possible to predict how TgCDPK1 regulates microneme secretion based on homology to model systems. The chemical-genetic methods we described represent an unbiased approach to identifying targets of TgCDPK1, and will hopefully reveal the pathways that lead to regulated exocytosis in apicomplexans.

Affinity for bulky-ATP analogues is conferred by an expanded ATP-binding pocket, which results from a small gatekeeper residue. We previously demonstrated that such an expanded ATP-binding pocket naturally occurs in TgCDPK1, rendering it susceptible to inhibition by PP analogues. In the present study we make use of a converse approach, which tracks the activity of a particular kinase through the use of a synthetic bulky ATPγS substrate (Fu-ATPγS) that can only be bound by kinases with an expanded ATP-binding pocket. We found that TgCDPK1 could use Fu-ATPγS to thiophosphorylate a heterologous substrate, in a manner dependent on its glycine gatekeeper, and this ability was lost by mutation of the gatekeeper to a methionine, despite previous observations indicating that such a mutation preserves general kinase activity (Lourido *et al*, 2010). By monitoring the thiophosphate-labeling of proteins in parasite lysates, we were able to show that a number of proteins were specifically thiophosphorylated by TgCDPK1, since labeling was almost completely lost in lysates from parasites expressing the methionine gatekeeper allele. Although it is possible that in vitro kinase reactions using lysates may not completely

capture the reality of subcellular interactions, this approach provides an unbiased method for identifying kinase targets of TgCDPK1.

The main obstacle to identifying the thiophosphorylated proteins is isolating them from the parasite lysate. We adapted two methods developed by others for the isolation of thiophosphorylated proteins: immunoprecipitation of the targets using a monoclonal antibody directed to the alkylated modification (Allen *et al*, 2007), and covalent capture of thiophosphorylated peptides following digestion of the entire lysate (Blethrow *et al*, 2008). Both techniques identified a number of targets specifically thiophosphorylated in the presence of the endogenous TgCDPK1 ($CDPK1^{G}$) but not in a strain carrying a methionine gatekeeper allele ($CDPK1^{M}$). However only six of the proteins were identified by both strategies, and we choose these for further investigation. Clearly, based on the pattern of TgCDPK1-dependent thiophosphorylation, these six proteins only represent a subset of the labeled proteins, and future studies will need to improve on the reliability of the identification strategies. This may be accomplished through quantitative mass-spectrometry approaches, like stable isotope labeling, which allow direct comparison of different samples. Additionally, the isogenic backgrounds used in this study could also be used to describe the effects of specific TgCDPK1 inhibition on a broader phosphoproteome, which may reveal further information on which targets are most significant *in vivo*. Despite our current limitations, we were able to demonstrate that both of the conserved putative targets of TgCDPK1 were only thiophosphorylated when TgCDPK1 was present in the lysates, thus validating the overall approach for the identification of kinase targets.

Motivated by previous reports linking DrpB with microneme and rhoptry biogenesis, we sought to further study its phosphorylation. We determined that DrpB phosphorylation occurred *in vivo*, and significantly increased following stimulation of microneme secretion in a manner dependent on TgCDPK1. Taken together these results strongly suggest that DrpB is directly phosphorylated by TgCDPK1 during microneme secretion, although the

function of this phosphorylation remains to be determined. Current approaches have not revealed a dominant phenotype for alleles of DrpB lacking the C-terminal domain predicted to be phosphorylated. However, future studies will need to test the function of these mutant alleles in the absence of the endogenous allele, since the phenotype of these mutants may have been masked by the endogenous copy. It is possible that the previously identified role in biogenesis of secretory organelles is only one of the functions of DrpB, and that following biogenesis it also plays an important role in microneme secretion. Although dynamins and related proteins are typically thought of as mediating endocytosis, evidence in is accumulating for a more complex role in the regulation of the vesicular trafficking dynamics. Inhibition or knockdown of dynamins has been shown to reduce regulated NK cell degranulation (Arneson *et al*, 2008) and insulin granule secretion (Min *et al*, 2007). More recently biophysical evidence suggests that dynamins may regulate the rates of vesicle recycling (Jaiswal *et al*, 2009) and the size of fusion pores (Anantharam *et al*, 2011), and together these two parameters may significantly restrict both the quantity and the size of exocytosed material. In *T. gondii* and other apicomplexans microneme secretion is thought to occur through the conoid at a narrow segment of the plasma membrane (Carruthers *et al*, 1999a), and we observed that micronemes accumulated in this region, following stimulation of secretion in the absence of TgCDPK1. If DrpB mediates membrane recycling and its rate is modulated by TgCDPK1 phosphorylation, reduced recycling could obstruct the fusion site and prevent other micronemes from being exocytosed. This model only serves to illustrate how an endocytic process may affect exocytosis, and more evidence will be needed to construct a plausible model for microneme secretion.

We have shown that the ATP-binding pockets of related kinases can be altered to render them susceptible to bulky ATP analogues, in order to identify the phenotypes they regulate (Lourido *et al*, submitted; Chapter 3). The present study suggests that a complementary approach is effective for identifying the targets of the mutant kinases,

potentially revealing other proteins involved in the pathways described by the inhibitor studies. The processes regulated by TgCDPK1 and related kinases are crucial for host-cell invasion, and therefore difficult to target by unbiased forward genetics. Five of the six proteins we have reliably identified as phosphorylated by TgCDPK1 in this study are annotated as hypothetical proteins, and have no assigned function, as does nearly half of most apicomplexan genomes. The approach presented here has the potential of uncovering novel components of essential cellular pathways, based on their phosphorylation by the kinases that regulate these processes.

MATERIALS AND METHODS

Parasite Growth and Selection

T. gondii tachyzoites were grown in human foreskin fibroblasts (HFF) cultured in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen) supplemented with 10% tetracycline-free FBS (HyClone), 2 mM glutamine, 10 mM HEPES (pH 7.5), and 20 μg/ml gentamicin ,as previously described (Lourido *et al*, 2010). When noted, parasites were selected using growth media containing any of the following: ATc (1 μg/ml; Clontech), pyrimethamine (3 μM; Sigma).

Plasmid and Strain Generation

The TgCDPK1 cKO was generated as described in our previous study (Lourido *et al*, 2010). The Δku80Δhxgprt background, referred to for clarity in this study as $CDPK1^{G}$ (provided by V. Carruthers, University of Michigan, USA), was used to generate the $CDPK1^{M}$ strain as previously described (Lourido *et al*, submitted; Chapter 3).

The tagging constructs for PRP-Ty and Ty-DrpB were directionally cloned PacI to AscI into *pLIC-YFP-HXGPRT* (provided by V. Carruthers, University of Michigan, USA). The 5'-UTR of DrpB was amplified (primers: 5'GCG TTA ATT AAC CTC TTG CCG GAG C and 5'GCG GCC GCG CCG AGA CGT CCT CGC GTT TGC CGT CAT CGA GCG GGT CCT GGT TCG TGT GGA CCT CCA TCT GCC GAA GAT TTC GGA GG) from RH genomic DNA and spliced at a NotI site with the DrpB cDNA amplified (primers: 5'GAC GGC AAA CGC GAG GAC GTC and 5' GCG GGC GCG CCT TAG TCG CTG AAC AGC GGA TTG TTC) from a library generated with the SMART cDNA synthesis kit (Clontech). The PRP 5'-UTR was amplified from RH genomic DNA (primers: 5'GCG TTA ATT AAT GCA GCT TCG TGC GCA GCT CGA AG and 5'CAC TGT TAT CCT CTA TTT TGA G) and spliced at a SacI site with the PRP cDNA amplified (primers: 5'ATT CTG CTC CAG CGG TAG CGT G and 5'GCG GGC GCG CCT TAA TCG AGC GGG TCC

TGG TTC GTG TGG ACC TCC TCT TCG TCA TCA TCT TCG TCC G) from the library described above.

The *DD-GFP-DrpB* construct was provided by M. Meissner (University of Glasgow, UK). The *DD-Ty-DrpB* construct was generated by introducing the DD into the Ty-DrpB construct described above in three steps: (*i.*) introduce an NsiI site upstream of the Ty tag by mutating with the QuikChange II Mutagenesis Kit (Agilent Technologies) with primers designed according to manufacturer's instructions (primers: 5'GAA ATC TTC GGC AGA TGC ATG TCC ACA CGA ACC AGG A and reverse complement), (*ii.*) amplify the DD from *DD-GFP-DrpB* (primers: 5'GCG ATG CAT GTG CAG GTG GAA ACC ATC TC and 5'GCG ATG CAT CTG CAT GCT AGG TTC CGG TTT TAG) and clone into the NsiI site, checking for directionality by restriction digest and PCR, (*iii.*) remove the NsiI site upstream of the Ty tag by mutating as above (primers: 5'ACC TAG CAT GCA GAT GGA GGT CCA CAC GAA CCA GG and reverse complement).

The TetON expression construct was generated as the Ty tagged DrpB construct above, but the DrpB 5'-UTR was replaced by the RPS12 promoter containing TetO sequences (van Poppel *et al*, 2006). Truncations of the DrpB cDNA were achieved by amplification with the appropriate primers (primers 5'GCG TCT AGA TTA CAG CGC CCT CGT GCC TGC GTC and 5'GCG TCT AGA TTA GCC TGC GTC TCT CTG CAG CTC for 117 and 121 amino acid truncations, respectively).

Stable lines carrying the tagged constructs were generated by selecting the transfected pool with the appropriate drug, and isolating individual clones by limiting dilution. Clonal lines were maintained under pyrimethamine selection to prevent loss of the tagged alleles.

Electron Microscopy

For ultrastructural analysis, TgCDPK1 cKO parasites were grown 72 h -/+ ATc and harvested prior to incubating them for 5 min in DMEM alone or supplemented with 3%FBS and 2% EtOH. Parasites were fixed in a freshly prepared mixture of 1% glutaraldehyde (Polysciences) and 1% osmium tetroxide (Polysciences) in 50 mM phosphate buffer at 4ºC for 30 min. The low osmolarity fixative was used to dilute soluble cytosolic proteins and enhance the visualization of cytoskeletal and conoid structures. Samples were then rinsed extensively in cold dH_20 prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella) at 4ºC for 3 hr. Following several rinses in dH_20, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA). To get a quantitative view of the apical ultra-structure samples were coded and apical sections were collected and scored, in a blinded manner by two independent experimenters, for conoid extrusion and the number of micronemes within an 800 nm radius of the apical end.

Protein Purification and Kinase Assays

Full-length TgCDPK1 was cloned and expressed as previously described (Lourido *et al*, submitted; Chapter 3). Reactions were performed in a 20 l volume containing 0.5 g enzyme, 2 g dMBP (Millipore), 0.005% Tween-20, 2mM K_2EGTA or 2mM CaEGTA, 10mM $MgCl_2$, 1mM analogue indicated (Biolog), 20mM HEPES pH7.5. Thiophosphorylation was allowed to proceed 30 min at 30°C. Laemmli sample buffer was added to each reaction and before resolving by SDS-PAGE and blotting. Prior to western analysis, total protein was imaged with SYPRO Ruby protein stain (Invitrogen) according to manufacturer's instructions.

Thiophosphorylation and Target Identification

Freshly lysed parasites were washed twice with ice-cold PBS and kept on ice until the kinase reactions were performed. Approximately 5 x 10^8 parasites were used in a final reaction volume of 200 l. Reactions were performed in 1X D-PBS (Gibco) containing 2mM K_2EGTA or 2mM CaEGTA (Molecular Probes), 10mM $MgCl_2$, 1% NP-40, 1mM GTP, 100mM ATP, 50 mM Fu-ATPγS, 1X Phosphatase Inhibitor Cocktails I and II (EMD), and a protease inhibitor cocktail (E64, 1 g/ml; AEBSB, 10 g/ml; TLCK, 10 g/ml; leupeptin, 1 g/ml; Sigma). Thiophosphorylation reactions were allowed to proceed for 30 min at 37°C. Debris was removed by centrifugation at 20,000 x g, 10 min, 4°C. Reactions were stopped by adding EGTA to 4mM and alkylation was performed by adding PNBM (Epitomics) to 1mM, 2 h at room temperature. At this point samples for western blotting were combined with Laemmli sample buffer and boiled.

Immunoprecipitation (IP) of thiophosphorylated proteins was performed as previously described (Allen *et al*, 2007). In brief, PNBM was removed by buffer exchange with PD-10 columns (GE Healthcare), eluting proteins in an IP buffer containing 75mM NaCl, 1% NP-40, 1mM EGTA, and 20mM HEPEs pH 7.5. Each sample was immunoprecipitated with 10 g rabbit anti-thiophosphate ester (RmAb 51-8; Epitomics) immobilized on Protein G sepharose (Pierce). Following extensive washes with IP buffer, proteins were eluted from the beads by treating them with 20mM DTT and 1% RapiGest (Waters). A portion of the eluted proteins were analyzed by SDS-PAGE and stained with Oriole fluorescent gel stain (Bio Rad), according to manufacturer's instructions. The remaining samples were analyzed by mass spectrometry as described bellow, following trypsinization.

Isolation of thiophosphorylated peptides was performed with samples that were not alkylated, according to the published protocol (Hertz *et al*, 2010; Blethrow *et al*, 2008). In brief, samples were digested with trypsin and added to100 l of iodoacetyl-agarose beads (SulfoLink gel; Pierce) in 100 l of 50% acetonitrile. Following overnight incubation,

rotating in the dark, the beads were loaded into a disposable column and washed with 2 ml each: water, 5M NaCl, 50% acetonitrile, and 5%formic acid in water. Peptides were eluted with 500 l of a 1 mg/ml solution of Oxone (Sigma) and concentrated with C_{18} Ziptips. Samples were analyzed by LC-MS/MS with an LTQ- Orbitrap Velos, with data searched using the ToxoDB 6.1 database of *T. gondii* proteins. All the mass spectrometry was performed at the Danforth Plant Science Center's Proteomics & Mass Spectrometry Facility (St. Louis, MO).

Sequence Analysis

To determine protein homology between different apicomplexan genomes we used OrthoMCL DB version 5, as previously described (Chen *et al*, 2006). Both DrpB and PRP were present in all the apicomplexan genomes used in the analysis: *Babesia bovis, Theileria parva, Theileria annulata Cryptosporidium muris Cryptosporidium parvum, Cryptosporidium hominis, Toxoplasma gondii, Neospora caninum, Plasmodium vivax, Plasmodium knowlesi, Plasmodium falciparum, Plasmodium chabaudi, Plasmodium berghei,* and *Plasmodium yoelii.*

Immunofluorescence Microscopy

Immunofluorescence staining was performed as described previously (Starnes *et al*, 2009) following permeabilization with 0.1% saponin (Sigma) with mouse-anti-Ty (mAb BB2; Bastin *et al*, 1996) and rabbit anti-ALD1 (Starnes *et al*, 2006), followed by Alexa488-goat anti-mouse IgG (Invitrogen) and Alexa594-goat anti-rabbit IgG (Invitrogen). Images were acquired in a Zeiss Axioskop fluorescence microscope equipped with a 63X 1.3 numerical aperture lens and an AxioCam MRm camera (Carl Zeiss).

Immunoprecipitation and Western Blotting

Immunoprecipitation of Ty-tagged proteins was performed following thiophosphorylation and alkylation as described above. Protein G sepharose (Pierce) was bound to mouse-anti-Ty (mAb BB2; Bastin *et al*, 1996) 1 hr, washed with IP buffer, and incubated with samples overnight at 4°C. Following extensive washes with IP buffer, Laemmli sample buffer containing 2-mercaptoethanol (3% final concentration) was added to each sample, before boiling for 10 min.

Lysates or equivalent from 1-2 × 10^7 parasites per lane were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with rabbit anti-TgALD1, rabbit anti-thiophosphate ester (RmAb 51-8; Epitomics), mouse anti-Ty (mAb BB2; Bastin *et al*, 1996) or mouse anti-GRA1 (mAb Tg17-43, kindly provided by Marie France Cesbron, Genoble, France). The signals were detected using IRDye 680CW conjugated donkey anti-rabbit IgG (LI-COR Biosciences) and IRDye 800CW conjugated goat anti-mouse IgG (LI-COR Biosciences) on the Odyssey infrared imager (LI-COR Biosciences). Images were processed and analyzed using the Odyssey infrared imaging system software.

Radiolabeling Assays

For *in vivo* labeling, extracellular parasites were incubated in phosphate free DMEM (Gibco) with 1 mCi [^{32}P] orthophosphate (specific activity, 8500 Ci/mmol; Perkin Elmer), for 1h at 37°C, 5% CO_2. Labeled parasites were resuspended in media alone or supplemented with 3% FBS and 2% EtOH, and incubated for 5 min at 37°C, before lysing and western blotting, following the procedures above. Radiolabel was imaged with an FLA5000 phosphorimager (Fuji).

Lytic Assays

Parasites were grown with ATc 48h prior to inoculation of confluent HFF monolayers in 96-well plates. Infected monolayers were kept in the presence of ATc for an additional 72 h, fixed with 70% ethanol and stained with 0.1% crystal violet (Sigma). Parasite growth led to loss of monolayer integrity as determined by absorbance at 570 nm using an EL800 plate reader (Bio-Tek). Values were expressed as means of four replicates from a representative experiment.

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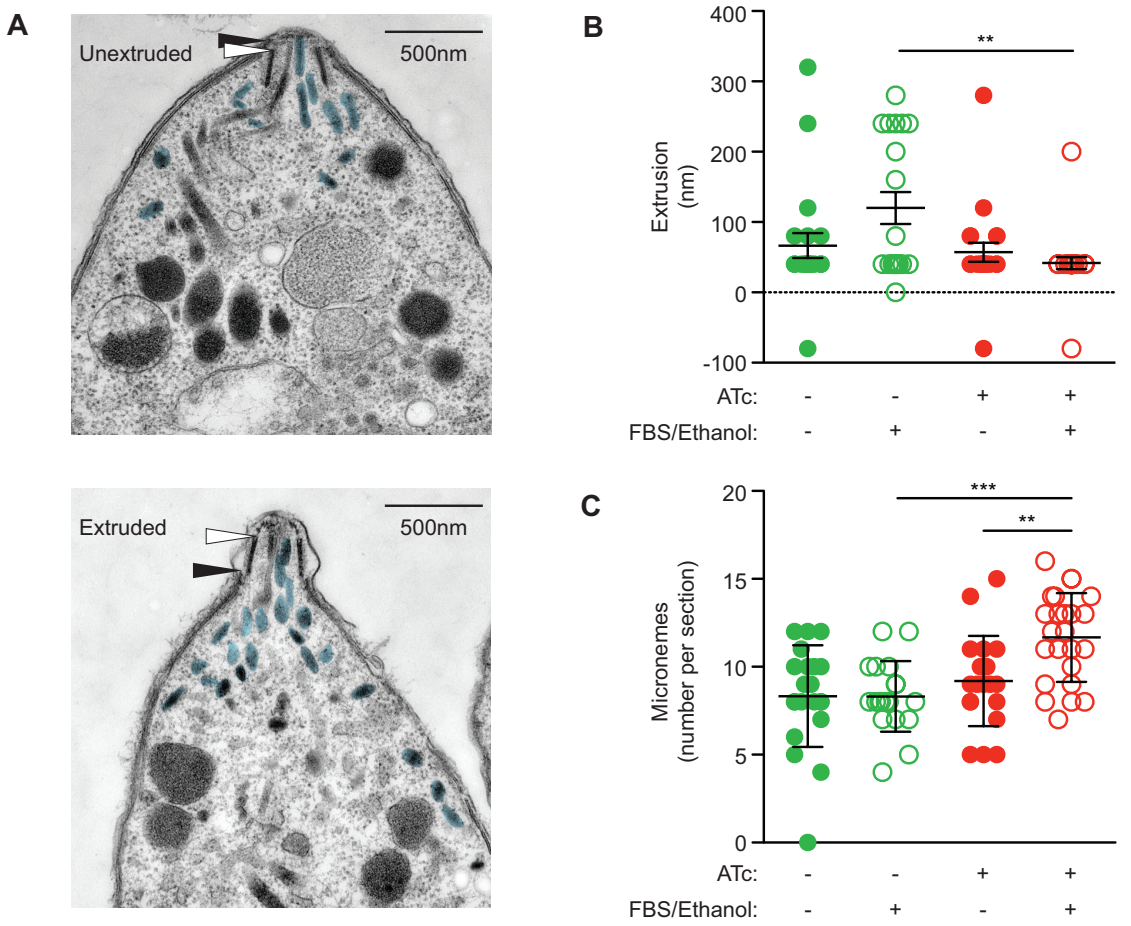

Figure 1. Regulation of apical morphology by TgCDPK1. The cKO parasites were grown -/+ ATc 72 h and treated 5 min with media alone or supplemented with 3% FBS and 2% ethanol (FBS/EtOH) to stimulate secretion. Apical sections of parasites were collected and scored in a blinded manner. (**A**) Representative sections for cKO grown without ATc, indicating the apex of the conoid (white arrow) and the apex of the IMC (black arrow), for parasites with unextruded (top) and extruded (bottom) conoids. Micronemes have been highlighted in blue. (**B**) Distance between the apical ends of the conoid and IMC, representing extrusion. Student's t-test; **, $P > 0.005$; means ± s.e.m., n = 19-24 sections. (**C**) Density of micronemes within an 800 nm radius from the apical end. Student's t-test; ***, $P > 0.0005$; **, $P > 0.005$; means ± s.e.m., n = 19-24 sections.

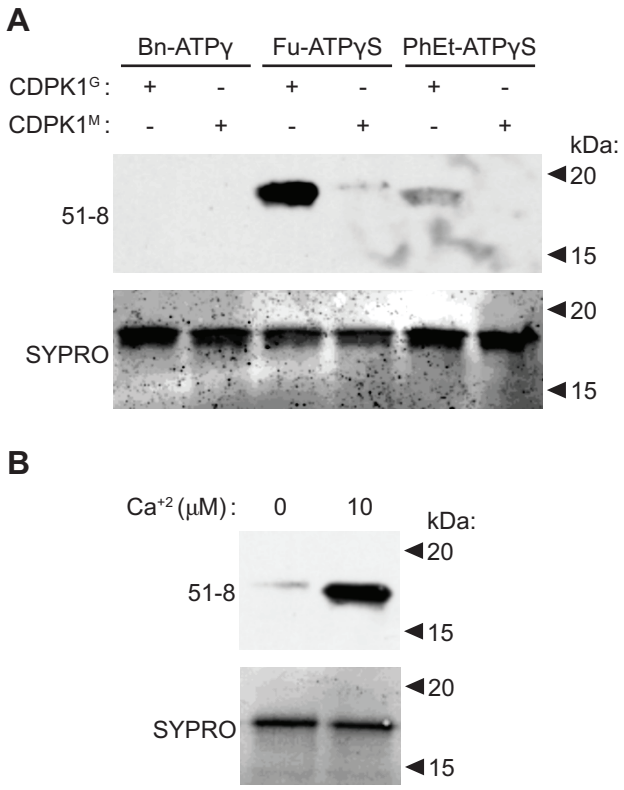

Figure 2. Thiophosphorylation of a heterologous substrate with different CDPK1 alleles. (**A**) Thiophosphorylation of dMBP by either widtype ($CDPK1^{G}$) or mutant ($CDPK1^{M}$) kinase using different ATPγS analogues. Thiophosphorylation was detected using a specific antibody for the modification (51-8) and total protein was stained with SYPRO Ruby. (**B**) Thiophosphorylation of dMBP by $CDPK1^{G}$ using Fu-ATPγS in the presence of either 0 or 10 mM free Ca^{2+}.

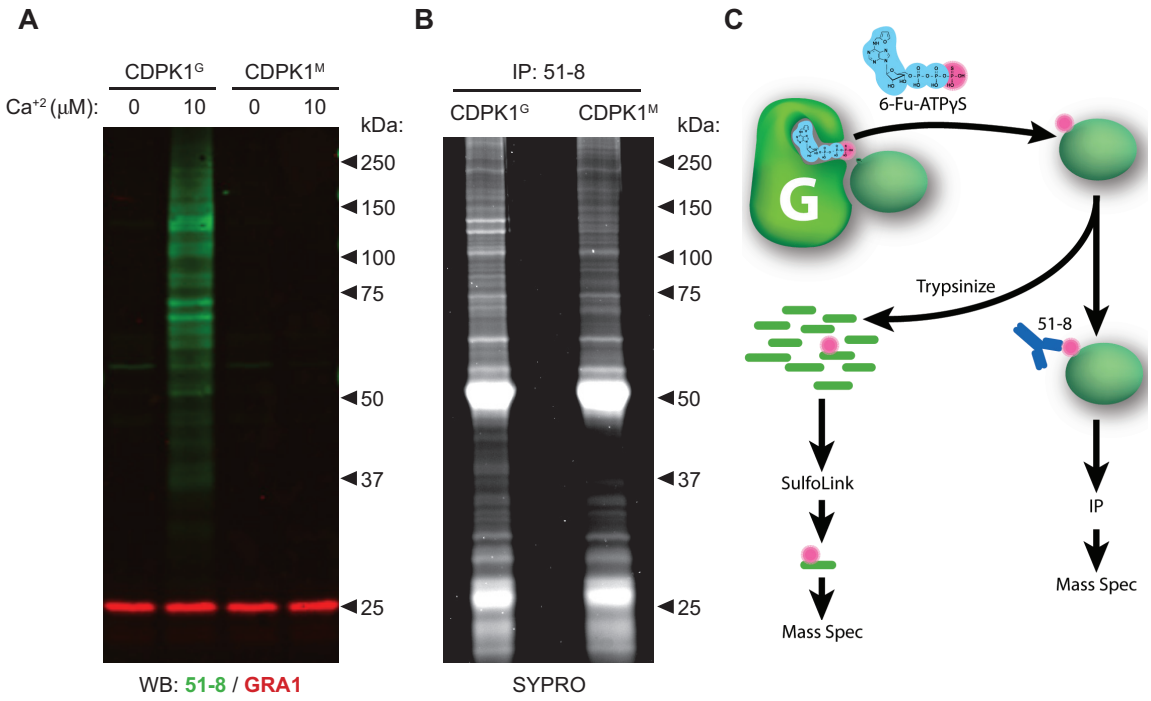

Figure 3. Thiophosphorylation and isolation of CDPK1 targets in *T. gondii* lysates. (**A**) Thiophosphorylated substrates in parasites expressing either CDPK1^{G} or CDPK1^{M}, in the presence of either 0 or 10 mM free Ca^{2+}. On the same blot, thiophosphorylation was detected with the specific antibody (51-8; green) and GRA1 (red) was used as a loading control. (**B**) Immunoprecipitation of thiophosphorylated substrates from parasites expressing either CDPK1^{G} or CDPK1^{M}. Total protein was stained with SYPRO Ruby. (**C**) Strategy for identifying thiophosphorylated targets. Fu-ATPγS is depicted in light blue with the g-thiophosphate, transferred to substrates, highlighted in pink. Targets can be either trypsinized to isolate thiophosphorylated peptides, or immunoprecipitated to isolate entire proteins.

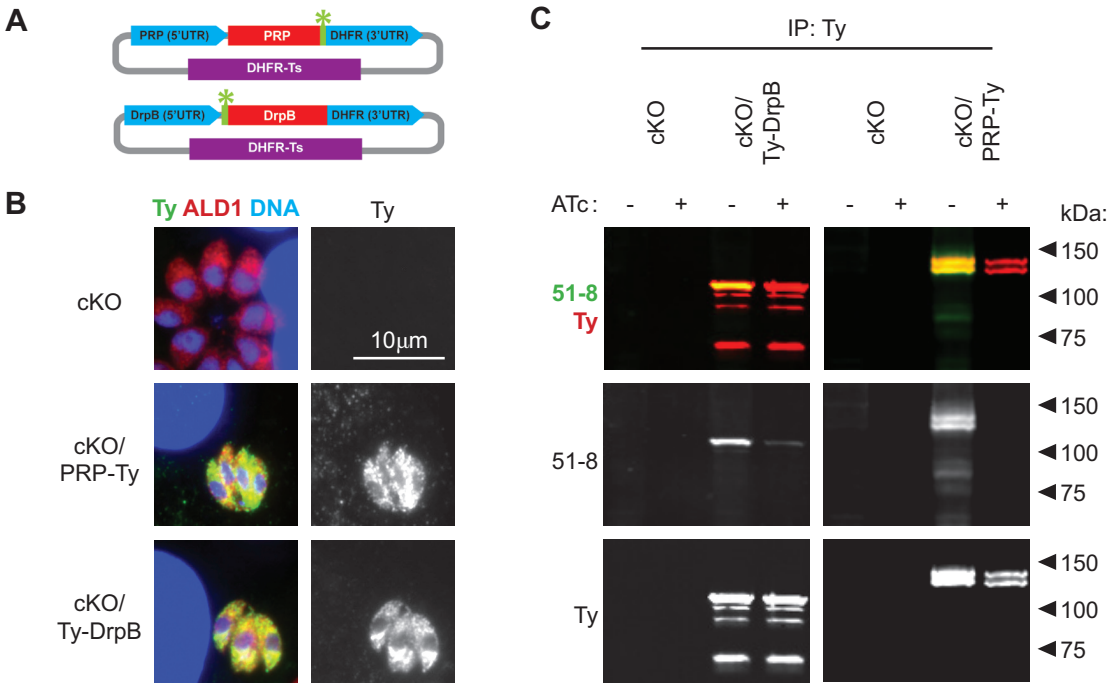

Figure 4. Putative targets are thiophosphorylated in a CDPK1-dependent manner. (**A**) Expression constructs for PRP and DrpB, cloned under their endogenous 5'-UTRs with either a C-terminal or N-terminal Ty tag (Green), respectively. (**B**) Expression of the tagged putative targets in the CDPK1 cKO strain. Intracellular parasites were probed for TgALD1 (red), Ty (green) and DNA (blue). All images were captured at the same magnification and the bar in the top right image gives the scale. (**C**) Tagged strains were grown 72 h +/-ATc. Following thiophosphorylation, putative targets were immunoprecipitated using the Ty-tag, and probed for either Ty (red) or thiophosphorylation (51-8; green).

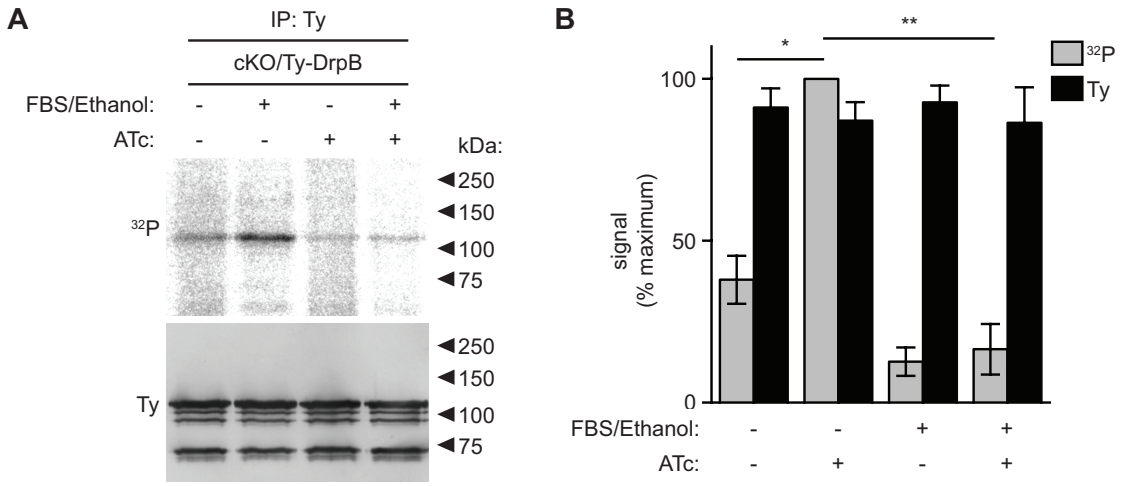

Figure 5. DrpB is phosphorylated *in vivo* in a CDPK1-dependent manner. (**A**) The TgCDPK1 strain expressing Ty-tagged DrpB was grown +/- ATc 72 h, incubated 1 h in media containing [^{32}P] orthophosphate, and treated with either media alone or supplemented with 3% FBS and 2% ethanol (FBS/EtOH). Parasites were subsequently lysed and immunoprecipitated DrpB was resolved by SDS-PAGE. Total DrpB was measured by WB (Ty), and radiation was measured by phosphorimaging (^{32}P). (**B**) Quantitation of WB and radiation signals, normalized to the highest signal in each experiment. Student's t-test; **, $P > 0.005$; *, $P > 0.05$; means ± s.e.m., n = 3 experiments.

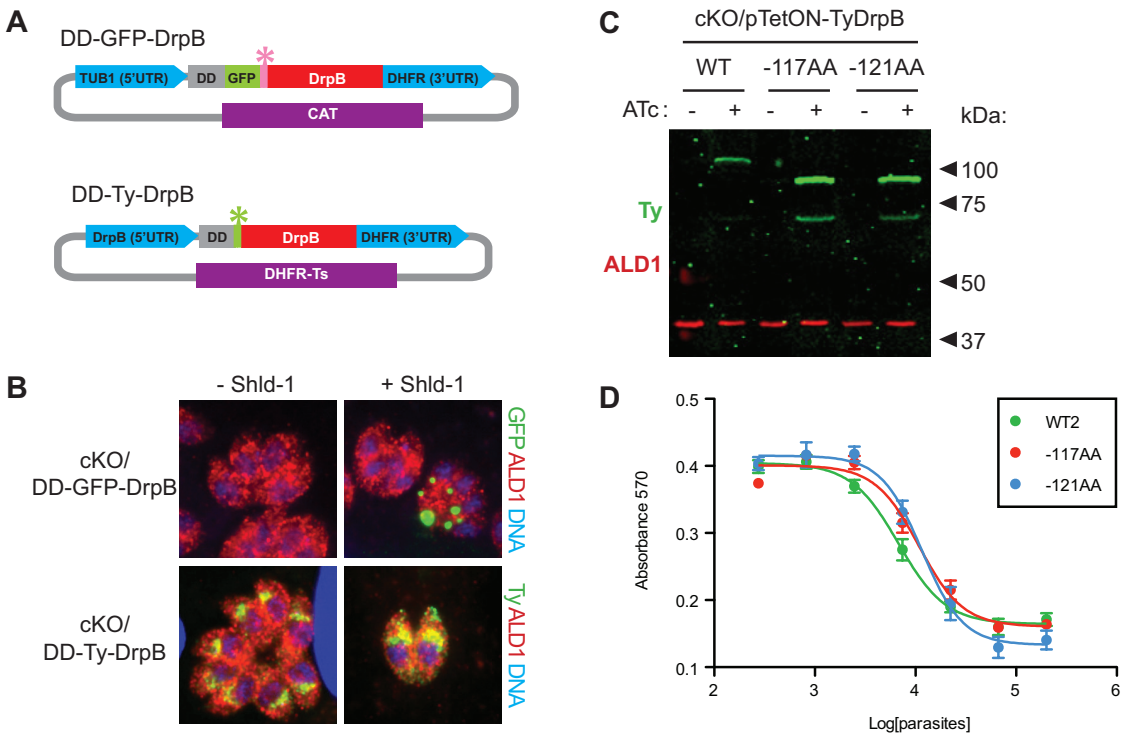

Figure 6. Attempts to determine the effects of DrpB phosphorylation on the *T. gondii* secretion. (**A**) Constructs used to express DD-tagged DrpB. Previously used construct (top) shows expression, under the TUB promoter, of DrpB N-terminally tagged with DD (gray), GFP (green) and c-Myc (pink). The new construct (bottom) is under the regulation of the endogenous DrpB 5'-UTR and DrpB is only tagged with DD (gray) and Ty (green). (**B**) Immunofluorescence microscopy of the TgCDPK1 cKO strain transfected with either construct, and incubated -/+ Shld-1 5h prior to fixation. Parasites were probed for TgALD1 (red), Ty (green) and DNA (blue). (**C**) Wildtype and C-terminally truncated alleles of DrpB were expressed under the TetON promoter. Lysates from strains incubated 48 h +/- ATc were resolved by SDS-PAGE and probed for Ty (green) and TgALD1 (red). (**D**) Strains expressing ATc-regulated alleles were incubated 48 h +ATc and used to infect HFF monolayers at increasing MOI. Monolayers were fixed and lysis of host cells was measured as a decrease in absorbance after crystal violet staining.

Gene ID	Annotation	MW (kDa)	Phosphopeptides[1]	Homology	
				P. falciparum	*C. parvum*
TGGT1_004440	Hypothetical	47	LGT*SGYGTYVGNGDDEQPTTTAGDAGVNR LGT*SGYGTYVGNGDDEQPTTTAGDAGVNRK	N/A	N/A
TGGT1_030680	Hypothetical	16	TDT*FVERAEELMNK TDT*FVERAEELMNK	N/A	N/A
TGGT1_064650	Dynamin-related	96	ALSSS*GVFDSKGASASAAK ALPNLQSFSS*FGSGEGR	PF3D7_1145400	CGD1_580
TGGT1_065330	Hypothetical	69	RLFT*FLQPDAPK LFT*FLQPDAPKR LFT*FLQPDAPK RLFT*FLQPDAPK LFT*FLQPDAPKR	N/A	N/A
TGGT1_088710	Hypothetical	86	LYS*HLSTGLKNSVSK LYS*HLSTGLK	N/A	N/A
TGME49_005320	Hypothetical	71	TVFESQKS*LTSTADFR TVFES*QPLQSR KDS*QTVFVSEPVSQSVAHFR TVFESQKS*LTSTADFR	PF3D7_0723300	CGD3_3900

[1] Observed phosphorylation sites are followed by an asterisk (*)

Table 1. CDPK1 targets identified by both direct peptide pulldown and immuno-precipitation.

Gene ID	MW (kDa)	Assigned spectra			
		Iodoacetyl pulldown		Immuno-precipitation	
		CDPK1^{G}	CDPK1^{M}	CDPK1^{G}	CDPK1^{M}
TGGT1_000530	17	0	0	3	0
TGGT1_002870	131	4	0	0	0
TGGT1_004440	47	2	0	2	0
TGGT1_006970	210	0	0	2	0
TGGT1_007360	76	0	0	5	0
TGGT1_007470	32	0	0	3	2
TGGT1_007720	148	5	0	0	0
TGGT1_008580	94	0	0	3	0
TGGT1_011620	69	0	0	2	0
TGGT1_014920	54	0	0	3	0
TGGT1_017550	26	2	0	0	0
TGGT1_018170	120	0	0	2	0
TGGT1_018920	87	0	0	4	0
TGGT1_020040	58	0	0	5	0
TGGT1_020820	15	0	0	2	0
TGGT1_021970	42	0	0	2	2
TGGT1_022040	60	0	0	0	2
TGGT1_022650	50	0	0	17	0
TGGT1_024480	22	0	0	3	2
TGGT1_028960	133	0	0	2	0
TGGT1_029230	34	0	0	4	4
TGGT1_029750	48	0	0	2	0
TGGT1_029790	15	0	0	3	0
TGGT1_030440	50	0	0	4	0
TGGT1_030680	16	2	0	3	0
TGGT1_030800	57	0	0	2	0
TGGT1_031600	58	0	0	5	4
TGGT1_031610	85	2	0	0	0
TGGT1_033520	37	0	0	3	0
TGGT1_036380	21	0	0	3	2
TGGT1_036680	70	0	0	2	0
TGGT1_037840	49	0	0	18	8
TGGT1_039300	17	0	0	2	2

Supplementary Table 1. (1/4) Proteins identified by mass spectrometry. The number of peptides found for each protein in each samples. Protein identified by fewer than two spectra with a confidence bellow 90% (Scaffold) were excluded from the analysis.

TGGT1_042150	203	0	0	0	3
TGGT1_042710	61	0	0	18	9
TGGT1_043160	60	0	0	3	5
TGGT1_043200	37	0	0	4	0
TGGT1_043965	16	0	0	2	0
TGGT1_045740	124	0	0	10	0
TGGT1_046000	84	0	0	3	0
TGGT1_047500	22	0	0	3	2
TGGT1_051010	65	0	0	4	0
TGGT1_051240	96	0	0	3	0
TGGT1_053260	52	0	0	3	0
TGGT1_056700	32	0	0	2	0
TGGT1_057800	37	0	0	3	0
TGGT1_062130	97	0	0	0	2
TGGT1_063360	76	0	0	2	0
TGGT1_063430	95	0	0	5	2
TGGT1_063520	42	0	0	6	0
TGGT1_063570	15	0	0	3	0
TGGT1_063760	62	0	0	3	0
TGGT1_064550	51	0	0	4	0
TGGT1_064650	96	2	0	11	0
TGGT1_065230	27	0	0	2	0
TGGT1_065330	69	5	0	4	0
TGGT1_065640	131	0	0	0	3
TGGT1_069300	53	0	0	0	2
TGGT1_071200	393	2	0	0	0
TGGT1_071440	22	0	0	3	0
TGGT1_072140	18	0	0	2	2
TGGT1_078300	221	0	0	5	0
TGGT1_080020	18	0	0	2	4
TGGT1_080510	52	0	0	6	0
TGGT1_081400	79	0	0	5	2
TGGT1_082670	24	2	0	6	4
TGGT1_083660	38	0	0	2	0
TGGT1_085550	50	15	2	19	5
TGGT1_085770	12	0	0	6	6

Supplementary Table 1. (2/4) Proteins identified by mass spectrometry. The number of peptides found for each protein in each samples. Protein identified by fewer than two spectra with a confidence bellow 90% (Scaffold) were excluded from the analysis.

TGGT1_085780	20	0	0	2	0
TGGT1_086220	93	0	0	6	0
TGGT1_086890	107	0	0	6	0
TGGT1_087090	35	0	0	5	0
TGGT1_087970	73	0	0	6	5
TGGT1_088710	86	2	0	2	0
TGGT1_092700	85	0	0	4	0
TGGT1_094210	50	0	0	3	0
TGGT1_098270	49	0	0	3	0
TGGT1_098470	100	0	0	0	2
TGGT1_099170	79	2	0	0	0
TGGT1_099280	24	0	0	12	0
TGGT1_100850	47	0	0	2	3
TGGT1_101070	136	0	0	23	0
TGGT1_101590	35	0	0	2	0
TGGT1_102900	66	0	0	2	0
TGGT1_103910	145	0	0	3	0
TGGT1_106270	66	0	0	2	0
TGGT1_107390	54	0	0	3	3
TGGT1_108230	94	0	0	2	0
TGGT1_108910	17	0	0	3	2
TGGT1_109620	83	0	0	2	0
TGGT1_112260	89	0	0	3	4
TGGT1_112840	72	0	0	3	0
TGGT1_114810	29	0	0	3	0
TGGT1_115350	29	0	0	2	0
TGGT1_115730	23	3	0	0	0
TGGT1_116480	51	3	0	0	0
TGGT1_120690	23	0	0	4	2
TGGT1_121340	35	0	0	2	0
TGGT1_121730	38	0	0	0	5
TGGT1_123210	53	0	0	0	3
TGGT1_123660	59	0	0	3	0
TGGT1_125220	31	0	0	4	0
TGGT1_126410	25	0	0	2	0
TGME49_002840	105	0	0	2	0

Supplementary Table 1. (3/4) Proteins identified by mass spectrometry. The number of peptides found for each protein in each samples. Protein identified by fewer than two spectra with a confidence bellow 90% (Scaffold) were excluded from the analysis.

TGME49_005320	71	4	0	2	0
TGME49_009980	62	0	0	6	4
TGME49_054370	477	2	0	0	0
TGME49_062050	65	0	0	0	2
TGME49_079100	48	0	0	2	0
TGME49_079380	35	0	0	2	0
TGVEG_030040	62	2	0	4	2
TGVEG_050040	64	0	0	2	2
TGVEG_105560	238	2	0	0	0

Supplementary Table 1. (4/4) Proteins identified by mass spectrometry. The number of peptides found for each protein in each samples. Protein identified by fewer than two spectra with a confidence bellow 90% (Scaffold) were excluded from the analysis.

CHAPTER V

Conclusions and Future Directions

INTRODUCTION

Apicomplexans are obligate parasites of animals, and rely on a specialized mode of motility to invade host cells and penetrate biologically restrictive barriers. With the exception of *Theileria spp.*, discussed in the introduction, all apicomplexan parasites of vertebrates perform this type of motility, referred to as gliding motility, because it is substrate-dependent and does not require membrane deformation or mobile appendages. Parasite stages that perform gliding motility are highly polarized, harboring specialized excretory organelles at their apical end and a network or vesicles, termed the inner membrane complex (IMC), subtending nearly the entire plasmalemma. During motility, adhesins are secreted at the apical end and translocated to the posterior via actomyosin motors anchored in the IMC (Sibley, 2004). In *T. gondii* motility is known to be triggered by a variety of extracellular stimuli that act by increasing cytosolic calcium. Compounds that prevent this rise in calcium block motility, such as chelators that accumulate in the cytosol and inhibitors of channels that mobilize calcium. A major consequence of the increase in calcium is the release of adhesins from specialized apical organelles called micronemes. This process may be triggered artificially by calcium ionophores or ethanol, which is thought to activate phospholipase C and consequently the release of calcium from IP_3 regulated stores (Carruthers *et al*, 1999; Lovett *et al*, 2002). Microneme secretion therefore appears to exemplify in apicomplexans the ubiquitous process of calcium-regulated secretion, which in the human body governs diverse processes from neurotransmitter and insulin release to leukocyte degranulation.

Experiments with general kinase inhibitors have demonstrated that microneme secretion in apicomplexans requires kinases. In animal cells two families of calcium-regulated kinases participate in regulated exocytosis: protein kinase C (PKC) and related kinases, and calcium/calmodulin-dependent protein kinases (CaMKs). No clear evidence for PKC exists outside of animals, in contrast to CaMKs, which appear more broadly

distributed. In plants and ciliates a different family of calcium-responsive kinases is represented by the calcium-dependent protein kinases (CDPKs), which are characterized by a kinase domain followed by four EF domains. The analogy to CaMK activation by calmodulin (CaM) binding is unavoidable, and potentially reveals something about their common ancestry (Harper *et al*, 2004). However, CDPKs form a distinct clade of kinases that is absent from animals, and therefore are of potential interest as therapeutic targets (Harper & Harmon, 2005). Furthermore, given that no clear CaMK homologue is conserved across all apicomplexan genomes, we hypothesized that perhaps conserved calcium-regulated processes, like exocytosis, were regulated by CDPKs in apicomplexans.

Phylogenetic analysis of apicomplexan CDPKs revealed at least four conserved kinases in apicomplexans with the canonical domain structure, and others with different numbers of EF domains (Billker *et al*, 2009). Two of the canonical CDPKs showed the greatest level of homology across different species, and these are called TgCDPK1 and TgCDPK3 in *T. gondii*. Besides their phylogeny the two groups may be distinguished by the type of acylation predicted for their C terminus and the type of residue present at a position that governs the depth of the ATP-binding pocket, termed the gatekeeper residue. While members of the TgCDPK1 group are predicted to be myristoylated and tend to have smaller gatekeeper residues, those from the TgCDPK3 group are predicted to be additionally palmitoylated and have larger gatekeeper residues. At the onset of our studies, little was known about the cellular function of these kinases. In *Plasmodium berghei*, where some of them were dispensable for the asexual cycle and could be knocked out, their loss was shown to result in developmental blocks. Knockout of a non-canonical CDPK, homologous to TgCDPK6, was shown to be involved in sensing of heparan-sulfates by sporozoites (Coppi *et al*, 2007). Similarly, knockout of a canonical CDPK unique to *Plasmodium spp.*, called PbCDPK3 but unrelated to TgCDPK3, was shown to be important for ookinete motility (Siden-Kiamos *et al*, 2006). Of the conserved canonical genes, only the homolog

of TgCDPK1 had been studied and although its knockout was viable in asexual stages, it showed a defect in male gametocyte exflagellation, which precluded the determination of its role in later developmental stages (PbCDPK4; Ishino *et al*, 2006). In *T. gondii*, the only report suggesting a function for a CDPK, used a Staurosporine derivative (KT5926) that inhibited TgCDPK1 *in vitro* and blocked parasite motility *in vivo* (Kieschnick *et al*, 2001). However, these studies were limited by the potential lack of specificity of the inhibitor used, and the fact that newly preparations of the compound have failed to reproduce the previous results (C. Beckers, personal communication). Our studies therefore sought to determine the function of conserved CDPKs in *T. gondii*, and focused on the two most conserved of the kinases, under the assumption that they would play important roles in apicomplexan biology.

Predicting that CDPKs would be essential for the parasite life cycle, we attempted to generate conditional knockouts (cKOs) of them using a tetracycline-regulatable system (Meissner, 2002). Using this approach, we were successful in generating a TgCDPK1 cKO, but unable to do so for TgCDPK3. Characterization of the TgCDPK1 cKO revealed that the kinase was dispensable for growth, but essential for microneme secretion. Consequently, loss of TgCDPK1 blocked all the processes associated with microneme secretion, including permeabilization of the parasitophorous vacuole membrane, motility, egress and host-cell invasion. The cKO could be rescued by complementation with a constitutively expressed wildtype allele of TgCDPK1, but not a kinase dead mutant, demonstrating that kinase activity is central to the function of TgCDPK1. Together these observations led us to propose that TgCDPK1 regulates calcium-dependent exocytosis in *T. gondii*, in a manner reminiscent of CaMK regulation of exocytosis in animals.

As mentioned above, TgCDPK1 and its homologues in other organisms have small gatekeeper residues, resulting in an expanded ATP-binding pocket compared to other kinases. In TgCDPK1, this residue is a glycine, which is unique among all active

T. gondii kinases (Peixoto *et al*, 2010) and generally rare in eukaryotic kinases. In yeast and mammalian cells, kinases engineered to have small gatekeeper residues have been shown to be sensitive to bulky pyrazolo [3,4-d] pyrimidine (PP) derivatives (Bishop *et al*, 2000). Predicting that these compounds would similarly inhibit TgCDPK1, we compared the sensitivity of the cKO complemented with either the wildtype kinase or a mutant where the gatekeeper had been changed to a methionine. Both kinases were equally capable of complementing the cKO, showing that kinase function was not impaired by the mutation. However, in the presence of the PP inhibitor 3-MB-PP1, parasites carrying the wildtype kinase were impaired in all the phenotypes associated with loss of TgCDPK1. In contrast, the mutant kinase was over 1000-fold less sensitive to 3-MB-PP1 than the wildtype kinase *in vitro*, and *in vivo* rendered parasites resistant to inhibition. These experiments demonstrated that TgCDPK1 could specifically be inhibited *in vivo* allowing us to regulate within minutes the activity of this essential kinase.

The observation that bulky PP analogues could be used to specifically inhibit *T. gondii* kinases opened up two new avenues of research: (*i*.) the possibility of specifically inhibiting other kinases, and (*ii*.) the identification of cellular targets of TgCDPK1. The former is conceptually identical to the experiments described above and requires a genetic background where TgCDPK1 is insensitive to the inhibitors and other kinases can be rendered sensitive through allelic replacement. The latter strategy makes use of artificial bulky ATP analogues that can only be used by kinases with enlarged ATP-binding pockets. This strategy was first demonstrated using [γ^{32}P] ATP carrying bulky substituents at the N^6 position (Shah *et al*, 1997), but has since been performed with similar ATPγS analogues, which has the advantage of generating thiophosphorylated proteins. Based on the specific reactivity of the sulfur group, thiophosphorylated targets can be isolated from lysates, enabling their identification (Allen *et al*, 2007; Blethrow *et al*, 2008). The potential for

understanding signaling pathways in parasites is clear, and encouraged us to validate both approaches in *T. gondii*.

The possibility of specifically inhibiting other parasite kinases was confirmed by the *in vitro* observation that TgCDPK3, which harbors naturally a methionine gatekeeper, is resistant to 3-MB-PP1, but can be rendered sensitive by mutating its gatekeeper to a glycine. To generate a genetic background resistant to bulky PP analogues, we replaced the endogenous allele of TgCDPK1 with one carrying a methionine gatekeeper. This manipulation was enabled by the recent development of *T. gondii* strains lacking the Ku80 component of the non-homologous end-joining DNA repair pathways, leading to increased rates of homologous recombination (Fox *et al*, 2009; Huynh & Carruthers, 2009). In this resistant TgCDPK1 background, we generated allelic replacements of TgCDPK3 either preserving the wildtype methionine gatekeeper or mutating it to the sensitive glycine allele, generating isogenic backgrounds in which to test the function of this kinase. By treating these strains with 3-MB-PP1 we were able to reveal that TgCDPK3 also regulates microneme secretion, although only under certain conditions. If the calcium concentrations of intracellular parasites are artificially increased with calcium ionophores, the parasites initiate motility and egress from host cells. However when TgCDPK3 is inhibited, the parasites fail to secrete their micronemes and remain intracellular, mimicking TgCDPK1 inhibition. A defect in microneme secretion was confirmed in extracellular parasites. However, in contrast to TgCDPK1, which was required for secretion under all conditions, inhibition of TgCDPK3 only affected secretion when triggered by both ethanol and fetal bovine serum (FBS), but not when triggered by ethanol alone. Although the signaling pathways regulated by FBS are unknown, reports from other organisms suggest that serum albumin, the most abundant protein in FBS, can trigger motility and microneme secretion (Kebaier & Vanderberg, 2010; Bumstead & Tomley, 2000). Furthermore two different pathways have been predicted to induce calcium release from intracellular stores: the

cyclic ADP ribose (cADPR) pathway and the IP_3 pathway stimulated by ethanol (Lovett *et al*, 2002). It is possible that both pathways are required for egress and that the cADPR pathway requires TgCDPK3 to function. This hypothesis could be tested by stimulating microneme secretion with abscisic acid, which has been shown to stimulate the cADPR pathway (Nagamune *et al*, 2008), and could lead to a better understanding of how these signaling pathways interact. Surprisingly, host cell invasion, which we have showed requires TgCDPK1, occurred independently of TgCDPK3, for the first time distinguishing the signaling pathways that regulate motility at these two points of the *T. gondii* life cycle. While investigating other pathways that lead to egress, we were also able to show that Zaprinast, an inhibitor of cyclic nucleotide phosphodiesterases that presumably leads to increases in cGMP, triggers egress in a process dependent on cGMP-dependent protein kinase (PKG). We also demonstrated that Zaprinast-induced egress could overcome the inhibition of TgCDPK3, suggesting that perhaps under certain conditions PKG activation might overcome the requirement for TgCDPK3 in microneme secretion. It has been shown in *P. berghei* that knocking out a phosphodiesterase can overcome defect in ookinete gliding motility caused by the loss of an unrelated CDPK (Moon *et al*, 2009), indicating that cyclic-nucleotide and calcium signaling pathways might converge on redundant functions, allowing different signals to regulate the same cellular process. Beyond the implications for understanding CDPK signaling in *T. gondii*, these studies represent proof of principle that this chemical-genetic approach provides a powerful means to study other parasite kinases. Additionally, this chemical biology method has the added advantage of allowing signaling events to be studied in a short time span, precluding the pleiotropic effects that may be caused by long-term loss of a kinase, a situation that may be unavoidable with certain genetic systems.

In order to further understand the signaling pathways regulated by TgCDPK1 we adapted the thiophosphorylation approach described above to parasites. We had observed

in vitro that wildtype TgCDPK1 could thiophosphorylate a heterologous substrate using a bulky ATPγS analogue (Fu-ATPγS). As predicted, the mutant version of TgCDPK1 harboring a methionine gatekeeper could not thiophosphorylate substrates using Fu-ATPγS, despite being able to phosphorylate substrates normally using ATP. To identify the targets of TgCDPK1 in parasite lysates, we compared wildtype parasites to those where the endogenous allele of TgCDPK1 had been replaced for one carrying a methionine-gatekeeper. We observed that robust thiophosphorylation occurred only in the presence of calcium and in the wildtype background, as would be expected for the targets of TgCDPK1. Two methods have been developed for the identification of thiophosphorylated proteins: immunoprecipitation using a monoclonal antibody specific to the modification, and direct capture of thiophosphorylated peptides using an iodoacetyl resin (Allen *et al*, 2007; Blethrow *et al*, 2008). We employed both methods, and identified the resulting proteins and peptides by mass spectrometry. A number of proteins were specifically identified in the samples coming from wildtype parasites. Encouragingly, only phosphopeptides were identified in the samples eluted from the iodoacetyl resin, as would be expected for the oxidized thiophosphate. Additionally, 18 different proteins were identified with at least two phosphopeptides each in the wildtype sample, and only a single one of these was identified in the mutant sample, as the only peptide found in that sample. Six proteins were identified by both methods, and of these two of them had homologues in other apicomplexans: a dynamin-related protein (DrpB) previously associated with the biogenesis of apical organelles including micronemes (Breinich *et al*, 2009) and a protein lacking any known domains but displaying a number of repeats with a periodicity of 28 amino acids. We could demonstrate, using tagged alleles of each protein, that both were thiophosphorylated by TgCDPK1. Furthermore, in radiolabeled parasites we could show that DrpB was phosphorylated *in vivo* during microneme secretion, in a TgCDPK1-dependent manner. Due to the lack of dominant phenotypes produced by mutation of the predicted phosphorylation

sites, or truncation of the C terminus of DrpB where they are found, we have been unable to confirm the role of these modifications in microneme secretion. Although the involvement of a dynamin-related protein might seem counterintuitive, given the documented role of dynamins in endocytosis, evidence is accumulating for the role of these proteins in the regulation pore size and duration during vesicular fusion(Jaiswal *et al*, 2009; Anantharam *et al*, 2011). These new functions have been proposed as explanations for the observed role of dynamins in NK cell degranulation (Arneson *et al*, 2008) and insulin release from pancreatic cells (Min *et al*, 2007). Further supporting a defect in vesicular trafficking during TgCDPK1 inhibition, ultrastructural analysis of the cKO showed that lacking TgCDPK1, following stimulation of secretion, micronemes accumulate at the apical end of the parasite. Although, our studies have yet to conclusively link DrpB to secretion, this is the first report of a validated CDPK target in apicomplexans and demonstrates the potential of this approach for illuminating these signaling pathways.

THERAPEUTIC POTENTIAL OF CDPK INHIBITION IN APICOMPLEXAN PARASITES

Serological surveys suggest that approximately 25% of the global population may be infected with *T. gondii* (Hall *et al*, 2001). During acute infection, parasites can disseminate throughout the host by traversing biologically restrictive barriers, which allows infection of difficult to treat areas like the central nervous system (CNS) and the eye (Barragan & Hitziger, 2008). However the vast majority of acute infections are self-limiting, induces the formation of tissue cysts that persist unnoticed for the life of the host. However, infection may recrudesce in immunocompromised individuals, leading to infections that are difficult to treat and frequently life threatening (Montoya & Liesenfeld, 2004). Available chemotherapy frequently causes adverse effects in patients, is ineffective in clearing chronic infection, and only marginally effective in treating CNS infection (McCabe, 2001). There is therefore a need to develop new chemotherapeutics against *T. gondii* and CDPKs as a whole have been considered attractive targets due to their absence from animal genomes and the drugability of kinases as a whole.

The realization that TgCDPK1 can be specifically inhibited by PP analogues has been followed up by a few other labs who have crystallized TgCDPK1 in the presence of some of these inhibitors (Ojo *et al*, 2010) and improved their specificity by screening derivatives of the PP scaffold (Murphy *et al*, 2010; Johnson *et al*, 2012). Despite the efficacy of these inhibitors against *T. gondii*, they have only been shown to have marginal efficacy in animal models of infection (Sugi *et al*, 2011). Our own work has recently revealed that this is at least partially caused by poor pharmacokinetics, which we are in the process of improving. Given the specificity of these compounds, and lack of inhibition in methionine-gatekeeper mutants of TgCDPK1, rapid development of resistance to this class of drugs is a concern. However because humans are a dead-end host for *T. gondii*, there is little risk of spreading resistant alleles that may arise under drug pressure. Furthermore, in our

own experiments we have failed to detect spontaneous resistance in tissue culture. These results suggest that studies to improve the *in vivo* properties of these compounds should be continued to fully assess their pharmaceutical potential.

The same compounds that inhibit TgCDPK1 have also been shown to inhibit its homologue in *Cryptosporidium*, which similarly has a glycine gatekeeper (Murphy *et al*, 2010). These results further encourage the development of these compounds for *in vivo* use, because of the lack of effective therapy against cryptosporidiosis in immunocompromised patients (Pantenburg *et al*, 2008). Like other apicomplexans, *Cryptosporidium spp.* use gliding motility to invade host cells (Wetzel *et al*, 2005), which relies on the calcium-regulated secretion of micronemes (Chen *et al*, 2004). In contrast to the systemic infections typical with *T. gondii*, *Cryptosporidium spp.* remain in the intestinal wall of the infected host, suggesting that inhibitors might require different pharmacokinetic properties, which might already be present in the available compounds. Although the limited growth of *Cryptosporidium spp.* in tissue culture precludes testing of a large number of compounds, the conservation of the kinases suggests that compounds could be initially tested in *T. gondii*, although the efficacy of currently available compound through the oral route should be determined. Our results, combined with their relative ease of synthesis, propose that these compounds could form a new class of antiparasitic drugs along with other drugs against essential parasite kinases (Kato *et al*, 2008; Donald *et al*, 2002).

BIOCHEMICAL DIVERSITY OF APICOMPLEXAN CDPKS

In plants, where they were first identified, CDPKs represent a prominent component of the kinome. In *Arabidopsis*, more than 42 isoforms grouped into 12 clusters have been identified, raising questions as to their specific regulation and diversity of function (Harper & Harmon, 2005). Expression profiling suggests that tissue specificity accounts for some of this diversity, with only one or a few representatives from most of the 12 clusters represented in a given tissue type (Harper *et al*, 2004). Biochemically, three other features have been proposed to account for the different roles of diverse CDPKs: subcellular localization, substrate specificity and calcium sensitivity (Harper *et al*, 2004). The extent to which each of these characteristics contributes to the functions of different CDPKs in apicomplexans still needs to be determined and would have important implications for understanding their signaling pathways and their adaptation to different cellular functions. We have already shown distinct subcellular localizations for the two related kinases, TgCDPK1 and TgCDPK3. As could be expected from their predicted N-terminal acylation, TgCDPK3 appears to be membrane associated and localizes to the cell periphery, while TgCDPK1 is more generally distributed. With the tools we have already developed, we could test the functional significance of their localization by mutating the sites predicted to be acylated. In TgCDPK3 we demonstrated that mutation of these sites led to differences in localization, but their functional consequences have not been assessed. A related question is whether an allele of TgCDPK3 mutated to have a similar localization to TgCDPK1, might be able to complement the TgCDPK1 cKO. This simple experiment would start to answer questions about the inherent specificity of different kinases for their substrates and the potential localization of those substrates. This analysis could be extended to kinases from other apicomplexans, which would inform whether they have similar substrate specificities and regulation.

The calcium sensitivity of different apicomplexan CDPKs also needs to be systematically assessed. Differences in calcium sensitivity may underlie differences between individual CDPKs and consequently represent differences in how they are regulated in the parasite. Importantly, it has been shown for plant CDPKs that substrate specificity may change under different calcium concentrations (Harper *et al*, 2004). Whether this occurs with TgCDPK1 could be tested following the approaches we developed to identify TgCDPK1 targets and could help us narrow down physiologically relevant interactions. The role of calcium in non-canonical CDPKs also remains unknown and should be determined, to establish whether these kinases also play a part in calcium-regulated processes or have evolved for different purposes. Associations between the calcium sensitivity of different kinases and the cellular functions they participate in may also help us define whether different calcium signals occur in apicomplexans leading to the activation of different CDPKs. It may also be important to consider whether differences in calcium oscillations may affect CDPK activity, acting as yet another layer of regulation as has been observed in CaMKs (De Koninck & Schulman, 1998)

Although our studies have failed to reveal a functional role for the presence of a glycine at the gatekeeper position of TgCDPK1, it remains an intriguing feature given its rarity among eukaryotic kinases and its conservation among homologous kinases in apicomplexans. Although individually, strains carrying wildtype and mutant kinases shown no growth difference, comparing their fitness in mixed cultures or *in vivo* may reveal subtle defects. We currently lack completely isogenic strains in which to test this, since we have relied on either their direct comparison in the cKO, or the comparison of wildtype with the allelic replacement, which lacks introns in a portion of the gene. However such strains could be generated and might reveal novel features of the regulation of TgCDPK1 and its homologues.

TAILORED INHIBITION OF *T. GONDII* KINASES

The *T. gondii* genome has 108 putative kinases and 51 pseudokinases, the latter defined by having an incomplete catalytic triad and therefore predicted to be inactive (Peixoto *et al*, 2010). This large number of kinases makes determining the contribution of individual kinases to signaling pathways a daunting task. Genetic manipulations can achieve specific disruption of a given kinase, but generally lack the temporal resolution required to assign kinases to specific pathways. In contrast inhibitors can frequently act in a manner of minutes but frequently lack the specificity necessary to distinguish between related kinases (Fedorov *et al*, 2007). We have demonstrated that, due to its atypical glycine gatekeeper, TgCDPK1 can be specifically inhibited by bulky PP analogues. By replacing the endogenous allele of TgCDPK1 with a mutant allele carrying a methionine gatekeeper, we were able to demonstrate the specificity of this class of inhibitors and generate a genetic background in which we could study other kinases. In this background, we replaced the endogenous allele of TgCDPK3, for one carrying a glycine gatekeeper and demonstrated the efficacy of this strategy for specifically inhibiting and uncovering the function of other *T. gondii* kinases. Because this strategy relies on the manipulation of a conserved domain common to all kinases, it represents in principle a generalizable approach that could help us unravel the function of the entire kinome.

Two major limitations preclude the global implementation of the chemical-genetic approach described above. The first is the observation, in other systems, that the small gatekeeper alters the catalytic properties (i.e., k_{cat} and $K_{m,\ ATP}$) of certain kinases, making them intractable by this approach (Garske *et al*, 2011). The second limitation, involves off-target effects of these compounds. Although during short incubations we have demonstrated that PP analogues are extremely specific to TgCDPK1, long-term growth in their presence causes toxicity, which is independent of TgCDPK1 and probably caused by secondary targets. Therefore, studying TgCDPK1 and TgCDPK3, both limitations were overcome

due to the tolerance to the gatekeeper mutations of both kinases, and the lack of side effects resulting from short incubations during the cellular assays involving egress, motility and invasion. However a global analysis of *T. gondii* kinases will require improvements to overcome both obstacles, which may be achieved by a new class of inhibitors and characterization of secondary targets.

In certain kinases, the deleterious effects of a small gatekeeper may be corrected by secondary mutations in the ATP-binding pocket (Zhang *et al*, 2005). Although the general location of these mutations may be predicted, final determination is usually empirical and requires the use of *in vitro* assays to measure kinase activity. This is laborious, but represents a viable alternative for many parasite kinases that can be produced recombinantly. Recently however, a new class of compounds has been developed that covalently inhibit kinases carrying a cystein at the gatekeeper position (Garske *et al*, 2011). These inhibitors displayed extraordinary selectivity in human cells as would be expected from the scarcity of kinases with cystein gatekeepers. More importantly, the authors showed that unlike mutation to a glycine, mutation to a cystein did not alter the catalytic properties of the kinase in question (Garske *et al*, 2011). Unlike the depth of the ATP-binding pocket, which may be expanded to different degrees by different amino acids, the ability of the nucleophilic inhibitors to covalently bind to a given kinase depends completely on the presence of a cystein at the gatekeeper position, and is therefore less prone to off-target effects. Analysis of the distribution of gatekeeper residues in *T. gondii* reveals that there are no active kinases with a cystein at that position. Therefore, nucleophilic inhibitors may be less toxic to parasites than the PP analogues we have previously used, which we have recently observed can also inhibit, albeit at higher concentrations, kinases carrying serine gatekeepers (unpublished). Reduced toxicity may facilitate longer treatments, which will be important to studies of kinases involved in parasite replication.

The availability of a second class of gatekeeper-inhibitor combination also opens the possibility of studies comparing the simultaneous, specific inhibition of two different kinases. Such an experiment may reveal whether two kinases are participating in the same pathway or in different pathways, by determining whether combined inhibition leads to additive or synergistic effects (Tallarida, 2006). The potential for this method may be better understood when considering our current knowledge of the regulation of microneme secretion. As demonstrated in chapter 3, inhibition of either TgCDPK1 or TgCDPK3 leads to a block in microneme secretion by intracellular parasites. Given this result, we cannot distinguish between two very different signaling arrangements: (*i.*) both participate in the same pathway required for secretion or (*ii.*) each participates in different pathway, but both pathways are required for secretion. Being able to inhibit the two kinases alone and in combination we could distinguish between the two possibilities, because the first would show additive effects of the two drugs, while the second would show synergistic. Such an analysis will be valuable, not only in conceptually understanding the signaling networks in the parasite, but also in making predictions about the targets of individual kinases, which will aid in identification, as discussed in the next section.

Use of the current PP analogues could be improved by generating a genetic background that is more resistant to long-term exposure to these inhibitors. The hypothesis driving these efforts would be that one or a few kinases, perhaps with alanine or serine gatekeepers, are being inhibited by bulky PP analogues, and if mutated to a resistant allele (i.e., methionine) would generate more resistant strains of *T. gondii*. There is a single kinase in the genome with an alanine at the gatekeeper position (TGME49_026540), and therefore predicted to be nearly as sensitive as wildtype TgCDPK1 to the inhibitors. This alanine-gatekeeper kinase is related to CDPKs, but lacks the calcium-binding domains. We replaced the endogenous allele of this kinase with methionine-carrying version, C-terminally tagged version, however the resulting parasites grew slowly, and did not exhibit increased

resistance to the compounds during long-term culture. This should be revisited, perhaps by expressing the kinase recombinantly, to address whether the methionine gatekeeper allele or epitope tagging changed activity or expression. With *in vitro assays*, we could investigate whether other gatekeeper residues are better tolerated or which compensatory mutations would be needed to preserve activity. Three other kinases are predicted to have serine gatekeepers and these include a conserved MAP family kinase (TGME49_112570), and two kinases (TGME49_039420 and ROP28) predicted to be secreted although they are not conserved among apicomplexans (Peixoto *et al*, 2010). The MAP family kinase is the most likely to affect growth if inhibited, but with only three candidates, allelic replacements are feasible. However if full resistance requires a combination of mutations, it might be challenging to develop a fully resistant strain through rational approaches. In this case, as with other drugs with unknown targets, chemically derived mutants could be selected for resistance. This strategy has been previously successful for isolating *T. gondii* mutants resistant to Artemisinin (Nagamune *et al*, 2007), calcium ionophores (Black *et al*, 2000), and cytochalasin D (Dobrowolski & Sibley, 1996). Although chemically induced mutations are generally difficult to detect, recent improvements and affordability of sequencing, have enabled complete genome sequencing of strains to identify mutations (Farrell *et al*, 2012). Taken together these approaches have the potential of improving the specificity with which we can inhibit kinases. The ability to perform long-term treatments would enable faster screening of strains for phenotypes associated with *in vitro* growth, and may permit inhibition of individual kinases in the context of a mouse infection. Finally, better knowledge of the secondary effects of PP analogues, would allow us to better assess their therapeutic potential, and determine their mode of action.

IDENTIFYING TARGETS OF CDPKS

Despite widespread interest in protein kinases and their targets, the incredible complexity and dynamic nature of cellular phosphorylation has limited our understanding of the signaling pathways that regulate individual processes. In human cells nearly a third of all intracellular proteins are phosphorylated, and frequently on multiple sites (Johnson & Hunter, 2005). Recently, the phospho-proteome of *T. gondii* has been described, and rivaling the situation in humans, ~24,000 phosphorylation sites were recorded on ~3,500 proteins, which corresponds to ~40% of all predicted proteins and ~67% of all proteins for which mass spectrometry data has been collected (Toxo DB). The complexity is daunting, but also represents an unprecedented opportunity to study kinase signaling, thanks to developments in mass spectrometry that enable monitoring of phosphorylation at such depth. Two major challenges remain to understanding and utilizing this wealth of data: attributing phosphorylation events to different kinases, and determining the presence and function of predicted phosphorylation events. In *T. gondii* the first of these challenges is addressed by the thiophosphorylation strategies we have used, although significant improvements are still needed to reliably identify thiophosphorylated proteins in a quantitative fashion. There are currently no high-throughput methods for validating kinase targets, however, we propose that comparing the targets identified by thiophosphorylation to quantitative phospho-proteomes may help select targets with higher confidence. Ultimately, these studies must culminate with a functional analysis of the phosphorylation sites described, which is a challenging standard, but a necessary one to establish biological significance. The regulation of gliding motility in *P. falciparum* presents a clear example of this, where analysis of phosphorylation in the tail domain of AMA-1, revealed that this modification was essential for invasion (Treeck *et al*, 2009). In contrast, phosphorylation sites on the motor complex, previously suggested to regulate assembly and function (Green *et al*, 2008), were recently shown to be irrelevant for this process (Ridzuan *et al*, 2012).

Currently our thiophosphorylation approach has relied on parasites expressing either wildtype or a methionine gatekeeper allele of TgCDPK1. In the future, we would like to expand this approach to other kinases, and presently the isogenic strains carrying TgCDPK3 with either a methionine or a glycine gatekeeper could be use for such a study. However our experience with isolating thiophosphorylated proteins suggests that there is a lot of variability in the quality of peptide isolation, and immunoprecipitation. Stable isotope labeling strategies have been widely used to provide relative quantitation, through the direct comparison of two samples by mass spectrometry (MS). The most common of these methods is stable isotope labeling by amino acids in cell culture (SILAC) where a sample grown with amino acids containing ^{13}C or ^{15}N, is compared with one grown in regular media (^{12}C and ^{14}N). The two samples are then combined and simultaneously analyzed by MS, and their peptides can be distinguished by the different masses of their constituent amino acids. This technique has recently been used to compare whole phospho-proteomes from different yeast samples (Dephoure & Gygi, 2011) and to compare thiophosphorylation in lysates from human cell expressing either wildtype or glycine-gatekeeper Erk (Carlson *et al*, 2011). An alternative strategy would be to control for total kinase activity internally within each sample, by adding both [g-^{18}O]-ATPγS, which could be used by most kinases, and the bulky ATPγS analogue (Fu-ATPγS), which is used exclusively by the glycine gatekeeper kinase. Although this method has not yet been attempted, a similar approach using [g-^{18}O]-ATP has demonstrated the feasibility of isotopic labeling of phosphate groups and subsequent analysis by MS (Zhou *et al*, 2007). The benefit of including [g-^{18}O]-ATPγS in the reaction would be that it could generate a baseline of possible thiophosphorylation to compare to the unlabeled thiophosphorylation specifically attributable to the glycine-gatekeeper kinase. Furthermore, we will get a quantitative sense of how much of the thiophosphorylation occurring at a particular site depends on the kinase of interest versus other kinases. In principle both SILAC and ^{18}O labeling could be combined to achieve

both internal comparison of specific activity to global activity, and external comparison of activity between two strains carrying different gatekeepers on the kinase of interest. This approach could also be used to identify thiophosphorylation by other kinases, dependant on the glycine-gatekeeper kinase, by comparing thiophosphorylation in the presence or absence of bulky PP analogues. Together, these methods will improve the reliability of target identification.

Our analysis of TgCDPK1 thiophosphorylation revealed that a few proteins from other compartments, like rhoptries and dense granules, which is to be expected from reactions in parasite lysates. These biologically irrelevant interactions may be excluded bioinformatically, although their presence reveals another area where improvements in the methodology could increase our confidence in the identified targets. Because of their charge, it is not possible to get bulky ATPγS analogue into live cells. Thiophosphorylation therefore requires cell lysis, which we have performed by detergent treatment. It is possible that other methods of cell disruption, like French press or hypotonic lysis, may preserve subcellular structures or protein complexes important for kinase function and regulation. These methods might increase the specificity of certain kinases while being compatible with the thiophosphorylation reaction. Although not currently available for bulky ATPγS analogues, caged nucleotides have been used in a variety of systems to load live cells. In these compounds the γ-phosphate of the nucleotide is esterified ("caged") to mask its charge and increase membrane permeability, and the cage can be removed by photolysis to produce the bioactive molecule (Ellis-Davies, 2007). Having overcome membrane permeability, caged nucleotides face other limitations, like degradation from nucleotidases and phosphotransferases, but these may be overcome with high enough concentrations enabling direct labeling of glycine-gatekeeper substrates *in vivo*.

Having demonstrated that at least for TgCDPK1 and TgCDPK3 we can replace the gatekeeper allele at the endogenous locus, we will be able to couple the thiophosphorylation

experiments described above with *in vivo* inhibition of the kinase of interest, and quantitative analysis of the phospho-proteome. We predict that the specificity of the inhibitors, coupled with current technologies for analyzing phospho-proteomes (Dephoure & Gygi, 2011), will enable us to, independently of thiophosphorylation, identify phosphorylation events attributable to the kinase of interest. Because many signaling pathways are connected *in vivo*, this method has the limitation of not being able to determine which phosphorylation events are directly catalyzed by a particular kinase. However, the intersection of targets identified by thiophosphorylation and phosphorylation events regulated by the kinase of interest, will generate a list of putative targets with the confidence needed to analyze their function.

Through the improvements that we have described, we hope that a larger number of kinases can be systematically studied in *T. gondii*, revealing both their function and their targets. In a manner analogous to forward genetics, identifying proteins regulated by a given kinase may help us understand their function, by linking them to specific pathways. Nearly 40% of all genes, and 70% of genes conserved uniquely within apicomplexans, code for proteins with unknown functions (Kuo & Kissinger, 2008). Our understanding of these organisms will therefore depend on uncovering the network of interactions that gives rise to their remarkable abilities.

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